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# THE DIFFERENTIATION OF TISSUE CELLS[1]

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## INTRODUCTION

One of the significant achievements of the experimental analysis of metazoan development is the concept of determination—that during embryonic differentiation the various organ and tissue primordia pass through stages of increasing specificity and stability until they finally form characteristic tissue types. Various experimental methods have demonstrated this increasing restriction of potencies to be a typical feature of the development of all metazoa, though the pace at which it is manifested may vary from group to group. Because of the stability generally demonstrated for tissues at advanced stages of differentiation, the conclusion has been drawn that the various tissues, *and their constituent cells*, are at this time irreversibly fixed. According to this point of view, once cells have differentiated into the characteristic histological types, fundamental changes in cell type are no longer possible (for example, thyroid epithelium to cartilage). Any changes in cell morphology or behavior that may be observed are considered to be minor, reversible changes within a cell type. Thus all modifications in cell form that occur during development may be fitted into one of two categories (Weiss, 1939, 1953). If they are stable and persist in the absence of the initiating stimulus they are considered to be "true differentiations" (irreversibly fixed). If on the contrary they are unstable, that is, do not persist upon removal of the initiating stimulus, they are termed "modulations."

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[2]My analysis of this problem has been greatly stimulated by discussions with a number of my colleagues; in this respect I am particularly grateful to Doctors Edgar Zwilling and Clifford Grobstein.

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These definitions have brought order into our thinking and have focused attention on differentiation at the cellular level. They have therefore materially influenced our approach to the problem of differentiation. The view that not all changes of cell character are of similar stability or of equal developmental significance has in particular had a salutary effect on discussions of this problem as in the interpretation of the morphological dedifferentiation that is routinely observed in tissue cultures (Champy, 1912) and in the formation of the regeneration blastema.

Although there have been voices urging caution (such as Bloom, 1937); Ephrussi, 1953; J. Needham, 1942; Spiegelman, 1948) and voices of protest (especially Harrison, 1933), the principle of irreversible differentiation has found widespread acceptance. It has in some instances even served as an established fact of development, upon which theorizing on the mechanism of cellular differentiation could be based (Darlington, 1948; Fischer, 1946; Lillie, 1929; Medawar, 1947; Monod, 1947; Powell, 1946; Rusch, 1954; Weiss, 1939, p. 468, 1950, 1953; but see Weiss and James, 1955). Sonneborn (1947) formerly belonged to this group but has since taken a more circumspect position (1949).

With any topic of such fundamental significance constant reexamination is necessary. It is the purpose of this paper to consider the present state of our knowledge of tissue cell stability. To this end we shall reevaluate the evidence upon which our thinking has been founded, ponder the significance of recent discoveries, and suggest general approaches for future investigation.

## CRITIQUE OF THE EVIDENCE

Certain difficulties in the determination concept were emphasized some years ago by Harrison (1933) in his famous treatise on this problem. In tests of the fixity of a primordium one frequently obtains different answers with different methods. In such instances it is proper to ask whether the causes of limited differentiation reside solely in the tissues, or in the imposed environment as well. Harrison further warned that the determination concept, resting as it does on negative evidence, is necessarily lacking in scientific certitude. "There is no way of finding out with certainty whether the particular quality which a cell seems to have is finally fixed, for there always may be new conditions, not yet tested, under which other potencies may be revealed." (1933, p. 318; see also Ephrussi, 1953, p. 99). Certainly in this, a fundamental problem of development, there is need for careful specification of the conditions of our experiments; and before generalizations can be accepted, plausibility must be fortified by rigorous examination of the results.

### *1. The Criterion of Self-Differentiation*

a) *The Evidence from Tissue Culture.* The presumed fixity of tissue cells rests almost entirely on the criterion of self-differentiation—the capacity of the part in question to carry through or preserve its type-specific

differentiation when isolated or explanted in a so-called neutral environment in vitro. [For Lillie (1929) this is the only acceptable criterion.] In most discussions of the fixity of differentiated tissue cells the demonstrated stability of certain tissues in long term tissue culture looms as the principal evidence (Ephrussi, 1953; Fischer, 1946; Lillie, 1929; Monod, 1947; Rhoades, 1943; Sonneborn, 1949; Weiss, 1939, 1944, 1949, 1950; Wilson, 1925, and others). It is necessary, therefore, to examine this work in some detail.

Although there have been many claims that differentiated tissues preserve their specific character in long term tissue culture, not all of these have been substantiated (cf. Bloom, 1937; Willmer, 1954). We will therefore concern ourselves only with cases whose validity is accepted, and which in turn have been most widely quoted.

Parker (1929) discovered that even though a tissue loses its distinctive histological character under optimal growth conditions in culture, it nevertheless betrays its source by its rate of outgrowth in standard chicken plasma-embryo-extract medium. Thus fibroblast cultures derived from periosteal tissue have a higher rate of outgrowth than those from embryonic heart. Furthermore, so-called pure cultures of these fibroblasts preserved their distinctive rates of outgrowth in vitro for 97 days (Parker, 1933). When heart fibroblasts were then cultured in a medium rich in embryo-extract, and periosteal fibroblasts were cultured in a medium poor in embryo-extract, the relative rates of outgrowth were reversed. But upon return to the original regimen, the tissues reverted to their original rates of outgrowth!

It seems indubitable that under stable growth conditions in culture these cell strains possess a degree of stability. But these results do not justify Parker's (1932) conclusion that "The organism is able to effect progressive and irreversible changes in the constituent cells." Leaving aside the questionable significance of small rate differences in so complex a process as outgrowth, let us re-examine the conditions of the experiment. Cells from a single tissue or organ are cultured in an isolated state in a standardized medium in which nutrient materials are derived from the entire embryo. If one wished to design an experiment which would protect a tissue from other histogenetic stimuli and preserve, as far as possible, the specific character of the cells, it would be difficult to improve on this model. Is it therefore surprising that under such conditions certain characteristics of a tissue are stable?

A further indication that this stability may be spurious stems from the observation that the activity of fibroblasts in culture varies with the age of the embryo; skeletal muscle fibroblasts from 17-day chick embryos have a higher rate of outgrowth in culture than those from 8-day embryos (Parker, 1932). Apparently under *in vivo* conditions there is a change in character of this tissue between the 8th and 17th days of development. However, there is no such change *in vitro*. Fibroblasts from 8- and 17-day skeletal muscle are found to preserve the rate of outgrowth possessed at the time

of explantation for as long as 104 days under standard culture conditions (Parker, 1932). Maintenance of tissue character in these studies seems to be due more to the stability and generalized character of the medium than to the stability of the tissue cells. Under these circumstances the tissue cells appear to be in a state of developmental arrest.

Other oft-quoted examples involve the restoration in culture of the characteristic differentiation of certain tissue cells after its loss under optimal growth conditions. Pigmented iris epithelium depigments during 8-10 passages in media rich in embryo-extract, but reforms pigment granules under poor growth conditions with removal of the embryo-extract (Doljanski, 1930). When anterior hypophysis is cultured at 37 degrees in a medium rich in growth factors, no eosinophil or basophil cells are formed; but when nutrients are reduced and the culture kept at a lower temperature (28°-30°), many cells appear with abundant eosin-staining granules (Kasahara, 1935). Similarly, sheet outgrowth of kidney epithelium redifferentiates into tubules, when a fragment of connective tissue is added to the culture (Drew, 1923).

In these and like studies a certain stability of the tissues under the imposed conditions is indicated. Certainly a partial redifferentiation of iris epithelium and hypophysis is clearly demonstrated. This is quite to be expected, however, inasmuch as it occurs under generalized conditions. Reducing the mitotic rate and particularly the cell movements merely creates conditions that, being more like those *in vivo,* encourage differentiation. In the absence of new influences the tissues apparently follow the path of least resistance, and redifferentiate according to type. It may be noted, incidentally, that even under these generalized conditions tissues do not always redifferentiate. If iris epithelium is cultured in such a manner that it becomes deficient in nutrients and oxygen, it may form keratin-like material (Fischer, 1924).

The formation of tubules by kidney epithelium when combined with connective tissue is less clear. This may not be a redifferentiation of kidney tubules at all, but rather a non-specific reaction of an epithelium to a change in its tissue associations (cf. Levi, 1934; Bloom, 1937). Iris epithelium, for instance, will form duct-like structures when combined in culture with heart fibroblasts (Ebeling and Fischer, 1922; Fischer, 1946, p. 225).

We must then conclude that while many studies of self-differentiation in tissue culture definitely demonstrate stability under the imposed conditions, these studies do not by any means provide final evidence for irreversible fixity of tissue cells. On the contrary, they present evidence that different in vitro environments may elicit different responses from cells. Stability of cell type in culture appears to depend upon stability of the environment.

b) *The Significance of Mass.* Our conclusions on cell potency have been based almost entirely on experiments involving tissue masses consisting of

many cells. A fallacy in these conclusions is revealed by the repeated observation that the degree and variety of differentiation obtained tends to vary directly with the mass of tissue involved (Andres, 1953; Berrill, 1945; Chalkley, 1945; Child, 1928; Galtsoff, 1925; Grobstein, 1952; Grobstein and Zwilling, 1953; Lopaschov, 1935). Thus explants of presumptive head mesoderm from a single early gastrula of *Triton taeniatus* form only muscle *in vitro,* but larger masses, consisting of identical cells derived from the fusion of several of these same primordia, form muscle, chorda, brain, sensory primordia and ectoderm (Lopaschov, 1935). In the reconstitution of masses of minced Hydra the number of hypostomes, feet, tentacles and cavities formed increases directly with increase in the mass of tissue (Chalkley, 1945). Apparently, with increase in number of cells, along with tissue mass, cell movements and interactions increase in complexity until at certain levels new capacities of cells emerge. Hence, a description of the potencies of a given tissue mass does not necessarily reveal the capacities of the individual constituent cells. This raises the possibility that when a tissue mass is judged to be determined by the usual criteria (for example, self-differentiation in explants and transplants) individual cells within the mass may retain wider potencies. It seems possible that in transferring a mass of cells to a new locus the immediate environment of the inner cells is but slightly altered. Such a slight change may be sufficient to effect a shift in cell differentiation during the earliest phases of development (as in early amphibian gastrula); but for later stages, generally considered to be fixed, a more acute change may be required. In such instances fixity of the mass could not be taken as evidence of cell fixity, and the status of cytodetermination would remain open.

c) *The Differentiation of Isolated Blastomeres.* Much thinking on the irreversibility of the determination process stems from studies on isolated blastomeres of eggs with so-called mosaic development (such as annelids, molluscs, tunicates). These studies, for example, form the main basis for Lillie's (1929) theory of embryonic segregation. The fact that the development of isolated blastomeres *in vitro* does not exceed their fate in normal development has been generally accepted as proof that an extraordinary degree of fixity is achieved before and during early cleavage in these eggs (Wilson, 1904 a, b; Conklin, 1933; Costello, 1945). That there is remarkable stability under the imposed conditions cannot be disputed; but again we may ask: is this due primarily to the state of the blastomeres or to the method of culture? The observation that in many instances blastomeres actually undergo less segmentation and differentiate more poorly in isolation than they do in the intact embryo gives weight to the suggestion (Costello, 1945, p. 53) that the culture conditions are not optimal for differentiation.

In all of these studies individual blastomeres are cultured in a relatively large volume of non-nutrient medium (approximately 0.5 cubic centimeter or more of sea water) in contact with air. Modern tissue culture methods

would suggest several improvements in this procedure. It now seems well established (Earle, Bryant, and Schilling, 1954) that a medium conditioned by unknown products of living cells is essential for the proliferation of single tissue cells or small clusters of cells in culture. This may be achieved by drastic restriction of the volume of the medium, so that the cells remain in association with their own metabolic products (Sanford, Earle, and Likely, 1948), or, more effectively, by the use of x-irradiated "feeder" cells (Puck and Marcus, 1955). Child (1928), Grobstein (1952), Grobstein and Zwilling (1953) and others have shown that the mass of a given primordium must exceed a given level if complex histogenesis is to occur in culture. A further factor influencing the differentiation of tissues in culture is the $CO_2$ tension in the medium (Spratt, 1949; Trinkaus and Drake, 1956). This suggests that the beneficial effects of metabolically conditioned media (see above) may be partly due to a rise in $CO_2$.

It seems likely that if these lessons were applied to the study of isolated blastomeres, more complete development would result. If the volume of the medium were greatly reduced (or several blastomeres cultured together), and the $CO_2$ tension raised, or if conditioned medium were provided by the method of Puck and Marcus (1955), increase in cellular proliferation may follow. By these means alone a state may be attained in which more complete differentiation is possible. We may also increase the degree and variety of differentiation by increasing the mass of a given type of protoplasm (cf. Lopaschov, 1935), perhaps by the aggregation of several blastomeres of one type, for example, several $1a^2$ micromeres. In view of the fact that the *in vitro* environment of an isolated blastomere in these experiments (sea water) is markedly different from its *in vivo* surroundings (other cells, intercellular material, colloidal material of the perivitelline space), attempts at nutritional enrichment of the medium are in order. This might prolong viability (generally low in these experiments), and thus increase the possibilities of differentiation (cf. Penners, 1937). Finally, following the lead of Yamada (1950) and Gallera (1953) (see below), chemical shock treatment of blastomeres may prove fruitful. This may disengage protoplasmic organization sufficiently so that when subsequently cultured other potencies may be revealed.

In summary, it seems possible that application of our improved knowledge of the requirements of tissues for proliferation and differentiation in culture to the classical problem of the mosaic egg may elicit different results. Until this is done our knowledge of the potencies of individual blastomeres of these eggs must be considered incomplete.

### II. *Cellular Transformations*

Thus far it has been our concern to demonstrate the inadequacy of the evidence upon which belief in the fixity of tissue cells has been based. Cogent though these objections may be, the most convincing evidence against cell fixity would be a positive demonstration of the transformation

of characteristic, cytologically differentiated cells into distinctly different cell types (metaplasia).

There are now a few studies in which just this has been shown to occur. It has been well-established for some years that upon removal of the lens from the eye of larval and adult newts a new lens may differentiate from pigmented marginal cells of the dorsal iris (for references see Reyer, 1954). Similarly, in Triturus, Stone (1950) has elegantly described the step by step transformation of retinal pigment cells into characteristic neural retina, complete with rods and cones. And now quite recently, Fell and Mellanby (1953) have demonstrated that in the presence of high levels of vitamin A body-wall skin of 6–7-day chick embryos will form ciliated, mucous-secreting epithelium in culture (see also Hopper and Mathews, 1953). This transformation is of particular interest. It suggests that vitamin A may be as important in the embryonic histogenesis of mucous membranes as it is in the maintenance of these tissues in the adult, and, as a defined chemical effect occurring in vitro, it is amenable to quantitative analysis. As a first step, Weiss and James (1955) have indicated that vitamin A has primarily an inducing rather than a supporting role, the induction occurring within a period of 15–60 minutes. This finding has considerable general significance, for it raises the possibility that the mechanism in this transformation, and in others as yet unanalyzed, is similar to that proposed a number of years ago for certain types of chemical carcinogenesis, where the combined necessity of "initiating action" and "promoting action" has been emphasized (cf. Berenblum, 1954).

Changes in cell type in the dorsal iris, the pigment layer of the retina, and in 6–7-day chick ectoderm are spectacular indications of pluripotency in tissues in advanced stages of differentiation, and as such administer a telling blow to notions of tissue cell fixity. In assessing their general significance, however, it must be established whether these are specialized, aberrant occurrences, or the first discovered examples of a widespread phenomenon. Certainly we can find no justification at this point for assigning the lability found in these particular cells to other types of tissue cells; but these studies nevertheless encourage us to raise the question of transformation of cell type as a possibility for all types of cells in all stages of differentiation.

In this context we have reason to focus our attention briefly on other instances of cell transformation. Although these involve cells in early stages of differentiation and are therefore less convincing for the argument, they serve to emphasize the possible general nature of the process. These studies are also of considerable methodological interest, employing defined chemical means to shift the course of differentiation. By exposing ventral marginal zone of early amphibian gastrulae to ammonia solution at pH 11.8–12.2 Yamada (1950) caused this material to form notochord, muscle and nephric tubules in culture. Controls, lacking the shock-treatment with high pH, formed blood islands, blood vessels, mesothelium and nephric tubules. An ingenious method of pretreating amphibian neurulae with urea

solution enabled Gallera (1953) to increase the competence of its otherwise unresponsive ventral ectoderm to neural induction, so that when grafted to the neural plate area it formed brain, optic vesicle, etc. A further sign of lability in the ventral ectoderm of the neurula is found in Wilde's (1955) demonstration that in vitro treatment of this layer with a presumed melanin precursor, phenylalanine, elicits the differentiation of characteristic dendritic pigment cells, like those of neural crest origin.

In addition to the aforementioned, in which changes in cell type are definitely demonstrated, there are a number of other studies in which the interpretation is not yet clear. Be that as it may; since they are suggestive of the possible general lability of tissue cells, they merit consideration. It was found some years ago that bone and cartilage formation may be initiated in the skeletal muscle of adult rabbits by implantation of periosteal tissue and alcohol extracts of bone (Levander, 1938). Although it has since been established that this is not a specific induction but rather a non-specific reaction to irritating substances (Heinen, et al., 1949), cellular transformation is certainly involved. The question is whether the bone or cartilage originates from fully differentiated tissue cells, such as muscle or connective tissue cells, or from more generalized, pluripotent cells.

The tendency of fibroblasts ("mechanocytes"—Willmer, 1945) to transform into macrophages (histiocytes) has been emphasized by a number of workers (cf. Bloom, 1937; Willmer, 1954). In some instances it hasn't yet been established whether the increase in number of macrophages is due to a cellular transformation or to a selective augmentation of the existing macrophage population (Chevremont, 1945, 1948). But even when morphological transformation to macrophage-type cells is observed directly (Weiss, 1944; Weiss and Wang, 1945), the significance of the change in cell form remains in doubt. Because of its generalized morphology, its ubiquity in the organism, and the relative ease with which other cells appear to assume its form, the macrophage presents an unusually difficult problem in histogenetic classification. We do not know whether to consider it a fully differentiated tissue cell, a morphologically generalized form of many types of tissue cells, or a histogenetically generalized, pluripotent cell. Depending upon which designation fits best, transformation of a particular type of cell into a macrophage would be, respectively, a change in specific cell type, a modulation, or a dedifferentiation.

The possibility that cell transformations play a role in pigment spread in spotted guinea-pig skin has attracted much attention of late (Billingham and Medawar, 1948, 1950), coming at a time when there is considerable interest in the role of plasmagenes in differentiation. These authors contend that pigment spreads from dark into white areas by the infective transformation of non-pigmented dendritic cells in the basal layers of the epidermis, rather than by pigment cell migration. In support of this hypothesis they have marshalled an impressive array of evidence. The argument has been greatly weakened, however, by the discovery (Billingham and Medawar, 1953) that the so-called white dendritic "cells" are in reality not located

in the basal layers of the epidermis, as are the melanocytes, but in more superficial layers. Nor has there been any attempt to test the hypothesis by depriving skin of non-pigmented dendritic cells (cf. Rawles, 1944). If the infective transformation hypothesis is correct this should prevent pigment spread. In view of these objections (see also Lederberg, 1952; Rawles, 1955) the mechanism of pigment spread in this material remains unknown. An interesting variation on the theme of infective transformation has been proposed by Niu and Twitty (1950) for the origin of post-metamorphic skin melanophores in Triturus. They suggest that these cells are derived from "macrophages," which in turn have acquired their pigment phagocytically from cytolyzing melanophores. This is an intriguing possibility. However, in view of the possibility that the trypan blue stained cells in their material are really melanoblasts of neural crest origin and the fact that no branched melanophore was observed to have acquired its pigment by ingestion, we must reserve judgement.

Although the problem of cell origin in regeneration and carcinogenesis is a primary stimulus for continuing interest in the potencies of tissue cells, more than the briefest consideration of these phenomena would be beyond the scope of this paper. The concept of irreversible differentiation requires that the regenerate originate either by type-specific redifferentiation of tissue cells, such as muscle from muscle cells or bone from osteoblasts, or from relatively undifferentiated, pluripotent cells called *neoblasts*, or from both of these sources. The occurrence of regeneration in forms with determinate, mosaic cleavage (cf. Harrison, 1933), and a wealth of provocative experimental work supporting the possibility that single tissues are the cellular source of the regenerate (summary in Needham, 1952) seriously shake the foundations of the redifferentiation and neoblast theories. Nevertheless, due largely to the extreme difficulty of devising critical experiments, the problem of cell origin in regeneration remains open. In carcinogenesis, the concept of irreversible differentiation requires that the cancer originate either from neoblasts, or by modulation. The independent stability possessed by many tumors upon repeated transplantation (for example, Jensen rat sarcoma) indicates that many cancers are not modulations. The results of extensive investigations of chemical carcinogenesis certainly weaken the neoblast theory; but they do not invalidate it. The neoblast is an elusive entity and not readily eliminated. As long as critical information on cell origin is lacking final judgement must be held in abeyance.

### III. *General Difficulties*

Finally, there are two additional difficulties in the concept of irreversible differentiation which, while not critical, call into question its usefulness as a research guide. In the first place, widespread acceptance of this concept has tended to discourage research on the potencies of tissue cells in advanced stages of differentiation. For if these cells are considered to be fixed, transformations of cell type are of course impossible, and all changes that occur must be modulations (even though the test of revers-

ibility is often not applied). Secondly, it has led to confusion in assessing the significance of cell changes in histogenesis. According to this point of view, most of the characteristic cytological and histological characters of the organism are mere modulations; they disappear when tissues are isolated and cultured under optimal growth conditions. This results in a tendency to consider striking differences in cell morphology and behavior *in vivo,* such as bone versus kidney, to be less important than slight and questionable differences *in vitro,* such as rate of outgrowth.

## CONCLUSIONS

It is clear that the evidence upon which the concept of irreversible differentiation has been based is inconclusive. The question of cytodifferentiation therefore remains open. Because of the incomplete nature of the evidence I take a frankly phenomenological position and define differentiation on the cellular level simply as an increase in the structural and functional specialization of cells. We cannot at present take a general position on the matter of cell stability.

This definition, although tentative and lacking in precision, has the advantage of reflecting the limited state of our knowledge of tissue cell stability. By implication there are several consequences of this. There is obviously a need for further research on the problem of cell transformation. In pursuing this subject, however, we have no reason to expect that all fully differentiated cells share a particular degree of stability. Different cell types may in fact vary widely, from extreme lability to complete fixity. It is conceivable, for example, that certain cell types may transform into almost any other type. For others, the instability may be limited to shifts in type within a histogenetic family (such as epithelia, nervous tissue, or mesenchyme and connective tissue). Some credence is given to this possibility by the occurrence of demonstrated and suspected transformations within such tissue families (Fell and Mellanby, 1953; Levander, 1938) and by the impressive evidence for nuclear differentiation recently presented by King and Briggs (1955). For still other cell types differentiation may be a truly unidirectional, terminal process, with only one type of end product possible. Perhaps this is true of presumed post-mitotic cells, such as neurones (but see Murray and Stout, 1947), erythrocytes, and neutrophils, and of certain tumor cell strains, such as Jensen's rat sarcoma.

In considering the mechanism of cell "transformation" we may be reminded that there are no compelling reasons for assuming that change of cell type must necessarily involve reversion to a more primitive stage in the normal differentiation of that type. Entirely new paths of differentiation may be followed. Another question of fundamental relevance to the mechanism of transformation concerns the role of cell division. Is cell division a prerequisite of transformation in any given instance? And finally, if lability is definitely indicated for a particular strain of cells, what are the control mechanisms that maintain these cells in a particular differentiated state during normal development and functioning? In brief, the potencies of

tissue cells, mechanisms for the transformation of cells, and mechanisms for the maintenance of cell stability must be worked out in each instance.

Cytodifferentiation must eventually find its explanation in terms of an interaction between intracellular and extracellular factors. Hence, speculations on the role of nucleus, cytoplasm, plasmagenes, induced enzymes, and the like in cellular differentiation usually find a receptive audience. It must be recognized, however, that our present ignorance of the stability of tissue cells limits the validity of many of these theories, especially those which assume the inexorable fixity of tissue cells. [The discussions of Spiegelman (1948) and Ephrussi (1953) are provocative exceptions in this regard.]

## PROSPECTUS

An attack on the problem of tissue cell stability must be founded upon the realization that apparent stability of these cells may not be due to their intrinsic properties but to properties of the tissue system as a whole. This may involve not only cell number and density, but the properties of the intercellular matrix (Grobstein, 1955). Consequently, in order to study the potencies of tissue cells they must first be released from their tissue system. There have been various attempts to achieve this by mechanical dissociation of the entire organism into cells and clusters of cells. (Brøndsted, 1936; Child, 1928; Galtsoff, 1925; H. V. Wilson, 1907; Weiss and Andres, 1952). These investigations have been useful in pointing the way, but the incompleteness of the dissociations, the extensive cell destruction, and the complexity of the cell suspensions have rendered the results difficult to interpret. The possibility of a more effective attack on this problem has emerged in recent years with the introduction of more subtle, chemical methods of dissociation. Holtfreter (1944) pioneered in this by using high pH to dissociate cells of early amphibian embryos. But this treatment seems to be ineffective with more advanced stages. The need for such an agent has been filled by the discovery that both trypsin (Moscona, 1952) and versene, ethylenediamine tetra acetic acid, (Zwilling, 1954) will cause many advanced primordia and tissues to dissociate into a suspension of individual cells possessing a high degree of viability. The use of trypsin as a dissociating agent has been discussed critically by Rinaldini (1954) and Harris (1955).

Once cells have been released from their tissue system, a first measure of their stability may be provided by their behavior as isolated cells in culture, and after aggregating with each other. Some of the first investigations of the behavior of isolated cells have revealed system-independent stability in isolated notochord cells (Mookerjee, Deuchar and Waddington, 1953) and heart muscle cells (Rinaldini, 1954; Cavanaugh, 1955). In those instances where the differentiation of a cell type is most readily described by its relations with other cells (for example, tubule cells) study of isolated cells is inadequate. Here we must allow cells of a particular tissue type to aggregate and reassociate. We may then observe whether they re-

constitute the original tissue system. When dissociated chick mesonephros cells are allowed to aggregate they form characteristic tubular tissue in culture (Moscona and Moscona, 1952; Trinkaus and Groves, 1955). Similarly, aggregates of dissociated wing mesenchyme may form hyaline cartilage and myoblasts (Moscona and Moscona, 1952). These results certainly indicate a remarkable capacity of these cells to reestablish their tissue system after dissociation. However, inasmuch as the dissociated cells were derived from the whole mesonephros, consisting of epithelia, connective tissue, and vascular tissue, and the whole mesodermal core of the wing bud, we cannot be certain that differentiation is entirely specific on a cell-to-cell basis, kidney tubules, for example, arising only from former epithelial cells. To provide critical evidence on cell origin in these aggregates it will be necessary to use cells from a single tissue (if possible) or devise methods of marking cells.

If we are to test effectively the capacities of tissue cells to change type, we must not only release them from their tissue system but must in addition expose them to new morphogenetic stimuli. Since the possibility of such stimuli being present in the preceding experiments is remote, we must seek other means. Though greatly limited by our poor understanding of the physico-chemical basis of cell organization and the mechanism of induction, and consequently confined to rather crude methods, we may nevertheless make a beginning attack on the problem. Once cells are released from the controls of their own tissue system by dissociation, they may be subjected to new controls by combining them in culture with dissociated cells of a distinctly different tissue, in a mixed cell aggregate. In such a complex aggregate the contact or close proximity of diverse tissue cells while they are differentiating could conceivably result in significant interactions.

In their important experiments with mixed aggregates of dissociated cells from various germ layers of amphibian gastrula and neurula stages Holtfreter (1944) and Townes and Holtfreter (1955) have observed differentiation of the different tissue types to occur regionally—epidermis and endoderm at the surface, neural and mesodermal structures inside. Townes and Holtfreter (1955) interpret these results as due exclusively to a sorting out of tissue-specific cell types by directed cell movements. They base this conclusion on the observation that the pigmentation of ectoderm cells makes it possible to distinguish them from other types, and on the assumption that cells of the neurula stage are "no longer responsive" to inductive influences (Townes and Holtfreter, 1955, p. 92). In view of our limited knowledge of potency on the cell level this assumption seems unwarranted. Accordingly, in aggregates of ectoderm and mesoderm where there is a demonstrated tendency for pigmented prospective epidermis cells to move outward and for non-pigmented mesoderm cells to move inward the differentiation of epidermis from ectoderm seems established, but the specific tissue fate of the individual mesoderm cells remains an open question. The possibility that there are changes in cell type in the mesoderm cells has not been excluded.

In our own approach to this problem (Trinkaus and Groves, 1955) we have employed tissues in more advanced stages of differentiation—3½ and 5-day chick mesonephros and wing bud. Mixed aggregates of cells from these organs differentiate into areas of mesonephric tubules, cartilage, loose mesenchyme, and keratinized epithelium, in characteristic regional arrangement. The results with amphibian material suggest that this regional differentiation may be due to directed cell movements. However, certain preliminary results suggest that transformations of cell type may also be occurring, namely, a tendency in our experiments for the younger stage tissue to predominate in mixed cell aggregates from tissues at different stages of development, and a direct correlation of the number of tissues differentiating with the size of the explant. It is apparent that the possibilities of the mixed aggregate method for studying the capacities of tissue cells are great. We may not only vary the tissues combined (cf. Wolff, 1954) but vary the relative number of cells and the relative degree of differentiation as well. But before definite conclusions can be drawn on the critical matter of cell origin we must devise an adequate means of marking cells, and reduce the number of tissue types involved. With a simple technique for production of cell clones available (Puck and Marcus, 1955), the possibility of dealing with pure strains of tissue cells now emerges as a practical possibility.

The efficacy of chemical shock treatment in increasing or preserving the potencies of embryonic cells (e.g., Yamada, 1950; Gallera, 1953) suggests an added approach that may prove rewarding. If dissociated tissue cells were subjected to such a treatment (cf. Weiss and James, 1955), it is conceivable that their type-specific organization would be so altered as to create greater responsiveness to new inductive influences. If these cells were then mingled with cells of another tissue type in a mixed aggregate, directed changes in cell type might occur. Although ignorance forces us to thread our way in a largely empirical manner, there is some reason to suspect that use of agents which interfere with protein metabolism would be fruitful. In view of the probability that the effect of urea on the competence of ventral ectoderm (Gallera, 1953; Gallera and Baud, 1954) is due to its well established activity as a mild depolymerizing agent for proteins (Neurath, et al., 1944), use of other such agents would be in order. Moreover, because of the close relation between protein and nucleic acid metabolism (Gale and Folkes, 1954), factors that interfere with the latter may also affect proteins. For example, Lallier (1954) has found that lithium chloride causes both a loss of ribonucleic acid and an inhibition of glycolytic activity in amphibian gastrulae. Presumably these metabolic effects of lithium are related to its effect on development. Probings such as these may open a wide and instructive area of investigation.

## SUMMARY

The evidence for the theory of irreversible differentiation of tissue cells is examined and found to be inconclusive. On the basis of this analysis:

a) another, more tentative definition of differentiation on the cellular level is proposed, and b) suggestions are made for future research on the stability of tissue cells.

## LITERATURE CITED


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# CARYONIDAL INHERITANCE AND NUCLEAR DIFFERENTIATION*

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Caryonidal inheritance of mating types in *Paramecium aurelia* was first reported by Sonneborn in 1937 and its relationship to problems of development was noted in 1947(b), but the significance of these observations and speculations has not been generally appreciated. Caryonidal inheritance implies that somatic nuclei may differentiate, that is, that nuclei with the same genetic elements are modified to control different phenotypes and that these nuclear modifications are "hereditary." This implication bears significantly on the problem of cellular differentiation, but has been largely neglected—chiefly perhaps because of a lack of corroborative evidence from other forms. The classical tests for nuclear differentiation during early stages of development were uniformly negative. Recently, however, strong evidence for nuclear differentiation at somewhat later stages has been provided through the work of King and Briggs (1955). It now appears that studies on caryonidal inheritance in the Ciliates may provide model systems of general interest.

The essential feature of caryonidal inheritance is the correlation between certain cytogenetic phenomena and the distribution of traits among the progeny of single conjugating pairs. To facilitate discussion these cytogenetic events and their associated terminology are presented diagramatically in an abbreviated form in figure 1. The details of these processes vary from species to species and those in figure 1 are based specifically on *Tetrahymena pyriformis* (Elliott and Hayes, 1953; Nanney, 1953; Ray, 1954). Conjugating pairs are formed when cells of diverse mating type are brought together under appropriate conditions. Following a series of prezygotic nuclear events (including meiosis, formation and transfer of gamete nuclei), syncarya of identical constitutions are established in the two members of a pair. The postzygotic nuclear events (involving nuclear division, differentiation and disintegration) result in the establishment in each exconjugant of two macronuclei and a single micronucleus. These two macronuclei are separated at the first cell division following conjugation and each of the cells produced at this division gives rise to a culture designated as a *caryonide.* The two caryonides derived from a single exconjugant are called *sister caryonides;* two caryonides from different members of

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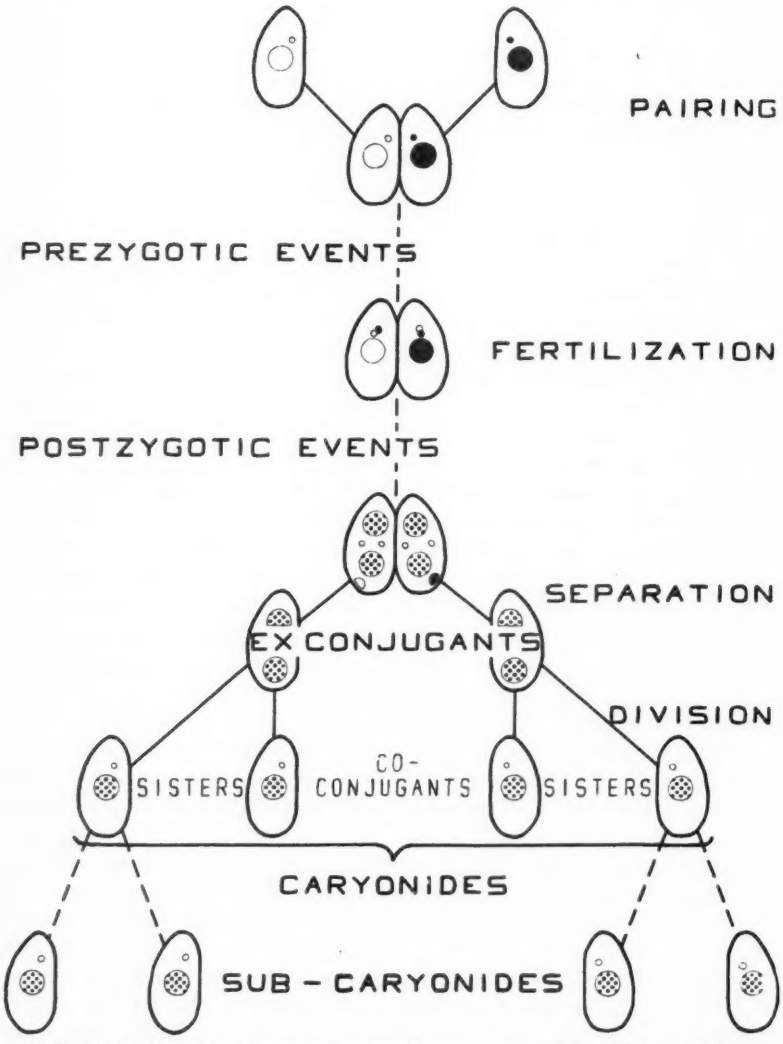


FIGURE 1. Abbreviated representation of events occurring during and after conjugation in *Tetrahymena pyriformis*. (See Text)

the same pair are called *co-conjugant caryonides*. Subcultures derived from a single caryonide at any time after its origin are designated as *sub-caryonides*. The nuclei in the cells derived from a single conjugating pair contain the same genes.

In spite of the genetic identity of the cells derived from a single pair, persistent mating type diversities appear among them. Caryonidal inheritance is indicated by a particular pattern in the origin of these mating type

diversities, that is, by a particular distribution of mating types among the cultures derived from single conjugating pairs. The details of this pattern vary from species to species, but one feature remains constant: sub-cultures of a single caryonide usually manifest the same mating types, while sister caryonides or co-conjugant caryonides often manifest different types. A high correlation is thus observed between the time of separation of new macronuclei (at the first cell division following conjugation) and the time when diverse cell lineages originate. The simplest interpretation of this correlation attributes mating type control to the macronuclei and implies that nuclei with the same genetic constitutions may differentiate so as to control different traits. Sonneborn (1947a, 1954) has presented several other lines of evidence which support this conclusion.

This paper documents certain features of caryonidal inheritance in variety 1 of *Tetrahymena pyriformis* and examines the significance of this system in assessing the role of the nucleus in cellular differentiation.

## MATERIALS AND METHODS

The strains used in this study were derived from two cells isolated by Elliott and Gruchy (1952) from a pond near Woods Hole, Massachusetts, and designated as WH-6 (mating type I) and WH-14 (mating type II). Crosses between these strains and among their progeny yielded a total of seven mating types which mate in all combinations of different types (Nanney and Caughey, 1953). The reactions among these seven types produce the breeding system shown in table 1.

TABLE 1

THE BREEDING SYSTEM IN VARIETY 1 OF *TETRAHYMENA PYRIFORMIS*. THE PLUS SIGNS INDICATE THE OCCURRENCE OF CONJUGATION; THE MINUS SIGNS INDICATE A FAILURE TO CONJUGATE.

| Mating Types | I | II | III | IV | V | VI | VII |
|---|---|---|---|---|---|---|---|
| I | − | + | + | + | + | + | + |
| II | | − | + | + | + | + | + |
| III | | | − | + | + | + | + |
| IV | | | | − | + | + | + |
| V | | | | | − | + | + |
| VI | | | | | | − | + |
| VII | | | | | | | − |

Upon inbreeding, the original strains gave rise to two diverse lines of descent, distinguished by the assortment of mating types they produce when crossed among themselves. These derived strains are designated as "Family A" and "Family B." Types I, II, III, V and VI appear in characteristic frequencies after conjugation within Family A; types II, III, IV, V, VI and VII occur regularly in Family B. A genetic analysis (Nanney, Caughey and Tefankjian, 1955) demonstrates that the distinctions between these families are due to differences in a single chromosome. Family A is homozygous for an allele, $mt^{(IV,\ VII)-}$ and Family B is homozygous for an

allele, $mt^{(I)-}$. Not only the mating type *potentialities*, but also the mating type *frequencies*, are strongly influenced by these genetic factors, as may be seen in the data tabulated in table 2. Although these effects appear to be related to a single chromosomal locus, sufficient data are not available to exclude several closely linked loci. The complexity of the differences in fact suggests a more complicated genetic basis.

Most of the methods used in this work have been presented elsewhere (Nanney and Caughey, 1955; Nanney, Caughey and Tefankjian, 1955) and will not be discussed in detail. It is necessary, however, to mention the methods used to establish that conjugation has in fact occurred. Some pairs separate without undergoing a complete nuclear reorganization, undergo no change in mating type, and do not enter a period of sexual immaturity. Screening for such pairs has been accomplished by several procedures, some of which are not satisfactory for an analysis of mating type distribution. Particularly, some crosses have been screened simply on the basis of the mating types of the derived cultures. If the two "caryonides"

TABLE 2

GENOTYPIC INFLUENCES ON MATING TYPE POTENTIALITIES AND FREQUENCIES IN VARIETY 1 OF *TETRAHYMENA PYRIFORMIS*.

| Genotype | Mating Type Frequencies | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | |
| $mt^{(I)-}/mt^{(I)-}$ (Family B) | .000 | .174 | .083 | .534 | .038 | .073 | .123 | 397 |
| $mt^{(IV,\ VII)-}/mt^{(I)-}$ (Hybrid) | .115 | .135 | .145 | .236 | .070 | .178 | .120 | 399 |
| $mt^{(IV,\ VII)-}/mt^{(IV,\ VII)-}$ (Family A) | .219 | .160 | .155 | .000 | .074 | .391 | .000 | 2350 |

from one pair member were of the same mating type as one of the parents and the two caryonides from the other pair member corresponded in mating type to the other parent, the pair was considered non-conjugant. It should be apparent that this technique eliminates non-conjugants (except in the unlikely event that one or both parents were unstable in their mating type [Nanney and Caughey, 1955]), but it also eliminates some of the conjugants and distorts the mating type distribution. Previously published data were taken from crosses which were not screened at all and are, hence, subject to a different kind of bias.

A more reliable procedure has been used exclusively in the experiments to be reported and is based on the observation that true conjugants are immature following conjugation. The duration of the immature state varies considerably depending upon the environmental conditions and the particular parents used in the cross, but in the crosses reported here no evidence could be found for true conjugants maturing before the 20th fission after conjugation. The maturity tests and associated manipulations are con-

ducted as follows. Conjugating pairs are isolated into depression slides. After 10–12 hours, when most of the pairs have separated and when none of the exconjugants has divided, the two members of a pair are isolated into different depression slides. During the next 12 hours these cells are periodically examined and the daughter cells are separated after the first fission. This procedure yields four depression cultures (caryonides) from each conjugating pair. Under our conditions (26 C. in Cerophyl inoculated with *Aerobacter aerogenes*) the cultures undergo 8–10 fissions in 24 hours and exhaust the medium on the second day, after having undergone a total of 12–13 fissions. These cultures are perpetuated by making single cell transfers to new depressions, usually on alternate days, until they have completed growth in seven depression cultures, and are then tubed. Those depression cultures remaining after new isolations have been made are designated as "left-over" cultures. The first left-over culture is tested for maturity.

Maturity tests are made by adding an approximately equal amount of a well-fed culture of a mating type unlike either of the parents and preferably of a type which the cross could not produce. On the following day the mixture has starved and the cultures have mated if they are mature. Those clones classified as non-conjugant on the basis of maturity tests are immediately tubed (from the second isolation culture) and tested for mating type, thus providing an additional test for non-conjugation.

One other source of possible bias must also be mentioned. Exconjugant cells are much smaller than vegetative cells and may remain quiescent for long periods of time. These cells often settle among the bacteria in the center of a depression slide and are difficult to find. Moreover, the time of division of the two exconjugants of a pair may differ by as much as several hours. It is conceivable that occasionally the products of a first division are confused with the exconjugants and that the subsequently separated "caryonides" are in fact sub-caryonides. This possibility of error is increased by the occasional occurrence of unilateral death in most crosses. The death of one of the fission products is also not a rare event and introduces a similar source of error. Although the *degree* to which these considerations may bias the results is not known, the *direction* of the bias is clear. Since sub-caryonides tend strongly to be alike in mating type, the mistaken identification of sub-caryonides as caryonides would produce an apparent correlation in the mating types of sister caryonides, even if none existed. On the other hand, this bias might eliminate a minor negative correlation among sister caryonides.

## RESULTS

1. *Mating type distribution.* The first question to be considered concerns the distribution of mating types among the cultures derived from a single pair. Ideally an analysis of mating type distribution would be accomplished by classifying each pair according to the mating types observed in the four caryonides, but the necessary number of pairs is not yet available. The

large number of mating types in this system gives a very much larger number of classes of pairs (120 distinguishable classes within Family A alone), some of which are reasonably common and some of which are very rare. Moreover, the death or loss of some of the caryonides in a pair renders that pair useless in such an analysis; many such "incomplete" pairs are available and should be used if possible. The data now available can be used to good advantage simply by making comparisons between paired cultures: sister sub-caryonides, sister caryonides and co-conjugant caryonides.

The most essential element in a system of caryonidal inheritance is the relative homogeneity *within* caryonides as compared with the diversity *between* caryonides. The distribution of mating types within sub-caryonides

TABLE 3

MATING TYPE COMBINATIONS IN PAIRED CULTURES FROM SINGLE CONJUGATING PAIRS IN FAMILY A

| Combinations | Sub-caryonides Observed | Sister Caryonides Observed | Sister Caryonides Expected | Co-conjugant Caryonides Observed | Co-conjugant Caryonides Expected |
|---|---|---|---|---|---|
| I–I | 20 | 20 | 20.9 | 39 | 39.5 |
| I–II | 0 | 31 | 33.4 | 69 | 64.0 |
| I–III | 4 | 32 | 34.1 | 42 | 51.3 |
| I–V | 3 | 13 | 12.7 | 25 | 24.5 |
| I–VI | 6 | 78 | 72.3 | 141 | 135.0 |
| II–II | 33 | 11 | 13.4 | 20 | 25.5 |
| II–III | 2 | 31 | 27.5 | 50 | 41.0 |
| II–V | 0 | 13 | 10.0 | 16 | 19.8 |
| II–VI | 1 | 59 | 57.7 | 110 | 109.0 |
| III–III | 30 | 22 | 14.1 | 19 | 16.6 |
| III–V | 2 | 7 | 13.2 | 17 | 15.8 |
| III–VI | 4 | 46 | 59.5 | 80 | 87.0 |
| V–V | 9 | 3 | 1.9 | 9 | 3.7 |
| V–VI | 1 | 20 | 22.0 | 33 | 41.8 |
| VI–VI | 34 | 68 | 63.2 | 120 | 115.5 |
| Totals | 149 | 454 | | 790 | |
| Chi Square | | 11.4 | | 6.5 | |
| D. F. | | 9 | | 9 | |
| P | | .2–.3 | | .7–.8 | |

derived prior to the 10th postzygotic fission is shown in table 3 for Family A and table 4 for Family B. Although differences occur within single caryonides and although many combinations of types occur, sister sub-caryonides are strongly predisposed to be alike. The observed differences are probably due to the occurrence of unstable caryonides from which different pure types have been derived (Nanney and Caughey, 1955). No striking tendency for any combination of types, other than like types, is apparent, but the data are not sufficiently numerous to exclude such a tendency and a thorough treatment of this problem will be deferred for later publication.

A comparison of the mating types of sister caryonides reveals no such tendency to identity (tables 3 and 4). The frequencies of the combinations

correspond well with the frequencies calculated on the basis of chance. These expected values were calculated as follows. The frequency of each type was derived from the entries in the column, thus yielding a frequency, $p$, for mating type I, $q$ for mating type II, $r$ for mating type III, etc. The probabilities for various combinations of types were derived from these frequencies; thus the probability for two mating type I cultures is $p^2$, for two cultures of mating type II is $q^2$, etc.; the probability for one type I and one type II is $2pq$, for one type I and one type III is $2pr$, etc. Although the

TABLE 4

MATING TYPE COMBINATIONS IN PAIRED CULTURES FROM SINGLE CONJUGATING PAIRS IN FAMILY B

| Combinations | Sub-caryonides Observed | Sister Caryonides Observed | Sister Caryonides Expected | Co-conjugant Caryonides Observed | Co-conjugant Caryonides Expected |
|---|---|---|---|---|---|
| II-II | 34 | 8 | 2.5 | 6 | 5.5 |
| II-III | 1 | 2 | 3.6 | 5 | 4.6 |
| II-IV | 2 | 27 | 26.2 | 37 | 34.2 |
| II-V | 0 | 0 | 2.0 | 1 | 2.6 |
| II-VI | 1 | 2 | 3.7 | 4 | 4.8 |
| II-VII | 0 | 7 | 6.0 | 7 | 7.9 |
| III-III | 9 | 2 | 1.1 | 4 | 1.4 |
| III-IV | 2 | 11 | 13.5 | 15 | 17.6 |
| III-V | 0 | 3 | 1.0 | 0 | 1.4 |
| III-VI | 1 | 1 | 1.9 | 2 | 2.5 |
| III-VII | 5 | 6 | 3.1 | 4 | 4.0 |
| IV-IV | 87 | 43 | 42.2 | 58 | 55.2 |
| IV-V | 0 | 5 | 6.5 | 7 | 8.5 |
| IV-VI | 6 | 11 | 11.9 | 12 | 15.6 |
| IV-VII | 7 | 18 | 19.3 | 23 | 25.0 |
| V-V | 5 | 0 | 0.6 | 0 | 0.3 |
| V-VI | 0 | 2 | 1.0 | 4 | 1.2 |
| V-VII | 0 | 1 | 1.5 | 4 | 1.9 |
| VI-VI | 4 | 2 | 1.1 | 1 | 1.1 |
| VI-VII | 3 | 3 | 2.7 | 6 | 3.5 |
| VII-VII | 14 | 1 | 1.8 | 2 | 2.8 |
| Totals | 181 | 155 | | 202 | |
| Chi Square | | 6.6 | | 5.3 | |
| D. F. | | 5 | | 5 | |
| P. | | .2-.3 | | .3-.5 | |

pairs used in this analysis were derived from crosses of clones from many different sources, the mating type combinations within pairs are essentially random.

An analysis of mating type combinations in co-conjugant caryonides produces similar results (tables 3 and 4). When all four caryonides are available, four different comparisons of co-conjugant caryonides are possible—each of the caryonides from each pair member with each of the caryonides from the other member. The first three comparisons, however, uniquely determine the fourth, and the first two comparisons partially determine the third. For this reason only two comparisons were made in tabulating the

data: one preselected caryonide from the first pair member with one preselected caryonide from the second, and the other caryonide from the first with the other caryonide from the second. Again close agreement is found between the frequencies of mating types observed and the frequencies expected. We are, therefore, led to the conclusion that the mating types developed by macronuclei within a single pair (or within a single exconjugant) are determined more or less independently.

TABLE 5

THE RELATIONSHIP BETWEEN THE MATING TYPE OF THE PARENT AND THE MATING TYPE FREQUENCIES IN THE PROGENY WITHIN FAMILY A

| Parents | Crosses | | Mating Type Frequencies | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | I | II | III | V | VI | Total |
| I, II | 6 | No. | 46 | 39 | 33 | 16 | 96 | 230 |
| | | % | .200 | .169 | .143 | .070 | .417 | |
| I, III | 2 | No. | 43 | 32 | 22 | 20 | 90 | 207 |
| | | % | .208 | .155 | .106 | .079 | .445 | |
| I, V | 2 | No. | 37 | 13 | 16 | 9 | 78 | 153 |
| | | % | .242 | .085 | .104 | .059 | .510 | |
| I, VI | 11 | No. | 106 | 70 | 90 | 37 | 165 | 468 |
| | | % | .227 | .150 | .192 | .079 | .355 | |
| II, III | 2 | No. | 37 | 22 | 18 | 3 | 83 | 163 |
| | | % | .227 | .135 | .110 | .018 | .510 | |
| II, V | 4 | No. | 38 | 24 | 24 | 19 | 57 | 162 |
| | | % | .234 | .148 | .148 | .117 | .358 | |
| II, VI | 7 | No. | 67 | 65 | 72 | 24 | 98 | 326 |
| | | % | .206 | .199 | .221 | .074 | .301 | |
| III, V | 3 | No. | 80 | 56 | 32 | 13 | 125 | 306 |
| | | % | .263 | .183 | .109 | .043 | .409 | |
| III, VI | 4 | No. | 28 | 22 | 24 | 16 | 54 | 144 |
| | | % | .194 | .153 | .167 | .111 | .375 | |
| V, VI | 6 | No. | 35 | 33 | 34 | 16 | 73 | 191 |
| | | % | .183 | .173 | .178 | .084 | .383 | |
| Total | | No. | 517 | 376 | 365 | 173 | 919 | 2350 |
| | | % | .219 | .160 | .155 | .074 | .391 | |

2. *Parent-progeny relationships.* The next question to be considered involves the relationship between the mating type of the parent and that of the progeny. In *P. aurelia* this problem can be explored by relatively simple procedures (Sonneborn, 1947a, 1950). Uniparental nuclear reorganization (autogamy) occurs periodically in all the stocks and presents no problem of interpretation regarding the mating type of the parent. Moreover, cytoplasmic "markers" are available which permit the identification of the cytoplasmic parent even when conjugation occurs in mixed cultures. Autogamy has not been found in *T. pyriformis*, however (though it occurs in a

related species, *T. rostrata* [Corliss, 1952]), and cytoplasmic markers are not yet available. Hence, less direct procedures must be used.

The method adopted was to make crosses among a series of cultures which differ in mating type and to examine the frequencies of the mating types among the progeny. If the parental mating type has an influence on the mating type of the progeny, significant and systematic differences in mating type frequencies would be expected in different crosses. The results of such crosses within Family A are shown in table 5. First of all, it is apparent that no *qualitative* distinctions are introduced as a result of variations in parental types; the same types appear among the progeny and in roughly the same proportions. Significant deviations do appear, however, in the relative frequencies of the types (P value of less than .001 for a chi square test for homogeneity). Certain patterns in the deviations can also be detected. Thus fluctuations in mating type III are approximately compensated by fluctuations in mating type VI; when types III and VI are combined into a single class, the homogeneity of all crosses is increased (P = .05-.10). These deviations, moreover, appear to be related to the appearance of these same types among the parents; when type III is in the parents, type III is usually depressed in the progeny; when type VI is in the parents, type VI is usually depressed among the progeny. These observations suggest a slight negative parent-progeny correlation, but the data are not convincing on this point.

The heterogeneity within table 5 may have been brought about by factors other than parental mating types. Although the parents were all homozygous for the $mt^{(IV,\ VII)-}$ allele, they may have become genetically diverse at other loci as a result of inbreeding. The crosses were performed with clones from several different inbred sub-lines and certain types of crosses occurred more often in some sub-lines than in others. Secondly, slightly different growth conditions were employed in some of these experiments and the treatments were not properly randomized. The progeny from some crosses were grown at maximal rate and yielded many selfers; other progeny were starved intermittently and produced largely stable cultures. The failure to control these variables was based on initial small scale experiments which showed no significant effect of these factors on mating type determination. In view of the slight deviations noted in the much larger samples, the initial experiments cannot be considered decisive and further studies will have to be undertaken to determine the cause of the variations. Modifying genes and environmental circumstances, as well as parental phenotypes, must be considered.

## DISCUSSION

1. *Patterns of Caryonidal inheritance.* Caryonidal inheritance of mating types has been demonstrated in two genera of the Ciliated Protozoa—Paramecium and Tetrahymena. Although agreeing in many essential features, various patterns of caryonidal distribution can be distinguished in these forms. The simplest pattern occurs in the Group A varieties of *P.*

*aurelia* (Sonneborn, 1937, 1947a; Kimball, 1937). Within a single variety only three classes of caryonides appear, those pure for one of the possible mating types, those pure for the other type, and unstable cultures which contain both mating types. Most of the study of this system has been concentrated on those strains and varieties in which mating type instability is rare or absent, but the available information on the other strains suggests no major modifications. The distinguishing features of this system are 1) a lack of any correlation between the mating types of parents and progeny, 2) a lack of any correlation between the mating types of sister caryonides or of co-conjugant caryonides, 3) a marked effect of temperature during the period of macronuclear development on the mating type frequencies, and 4) genetic control of mating type potentialities (Sonneborn, 1947a) and mating type frequencies (Butzel, 1955).

The observations on the Group B varieties of *P. aurelia* indicate the operation of a very different sort of system (Sonneborn, 1947a). The chief characteristics of this system are 1) a strong positive correlation between the mating type of the parent and that of its sexual progeny; 2) a strong positive correlation between the mating types of sister caryonides and a strong negative correlation between the mating types of co-conjugant caryonides when no cytoplasmic fusion occurs; 3) a strong positive correlation between co-conjugant caryonides when extensive cytoplasmic fusion occurs; 4) a slight temperature effect on mating type frequencies under normal conditions, but a marked effect when cytoplasmic fusion occurs (Nanney, 1954); and 5) no known effect of nuclear genes on mating type potentialities and only suggestive evidence for genetic effects on mating type frequencies. In spite of the differences between the Group A and Group B patterns, Sonneborn (1954) has clearly established that the macronuclei as certainly control the mating types in the B as in the A Group. The chief distinction between the groups is in the existence of a cycle of nucleocytoplasmic interaction in the B system; the macronucleus not only controls the mating type, but it is also responsible for establishing in the cytoplasm a mechanism for inducing new macronuclei to develop in the same manner as the old one (Nanney, 1954).

Studies on the system of mating type determination in *P. bursaria* are beset with many difficulties, but sufficient information is available to suggest a pattern of caryonidal inheritance related to that in the Group B varieties of *P. aurelia*, but involving multiple mating types (Jennings, 1942). The significant observations are as follows. 1) The progeny of a single pair usually have the same mating type, but when they differ, caryonidal distribution may occasionally be observed. 2) A cross of any two mating types *may* produce—in different pairs—all the mating types in that variety, but the parental types are usually in excess in the progeny. 3) Crosses of the same types derived from different sources may produce different spectra of mating types or the same types in different frequencies. 4) Cytoplasmic exchange probably occurs regularly during conjugation (Harrison and Fowler,

1945). The rarely observed differences between sister caryonides suggest, but do not unequivocally establish, caryonidal determination. The parent-progeny correlation suggests a cytoplasmic mechanism similar to that found in the Group B varieties, but modified by the regular occurrence of cytoplasmic exchange and the confluence of antagonistic cytoplasmic systems at the time of conjugation. The variations in crosses of strains from different sources suggest genetic determination of potentialities and frequencies. This interpretation of mating type determination in *P. bursaria* differs substantially from that proposed by Jennings, but is in essential agreement with a later interpretation by Sonneborn (1947a). One observation concerning the *P. bursaria* system (Jennings, 1941) is not precisely matched in the other systems and concerns the regular establishment of semi-stable conditions in which two types replace each other sporadically in the course of growth.

Finally, the caryonidal system found in Tetrahymena represents yet a different type of pattern, operating—like the *P. bursaria* system—with multiple mating types, but in a manner most similar to that in the Group A varieties of *P. aurelia*. No significant correlations have been established between sister or co-conjugant caryonides. As in the Group A varieties, a temperature effect (Nanney and Caughey, 1953) has been found on mating type frequencies and genetic influences on mating type potentialities and frequencies have been noted (Nanney, Caughey and Tefankjian, 1955).

2. *The nature and significance of nuclear differentiation.* That certain types of nuclear differentiation occur has long been established. Chromosome diminution, polyploidy, aneuploidy or polyteny are known to occur in a variety of tissues in many organisms. These nuclear alterations do not, however, occur in enough organisms and do not offer sufficient variety to play a predominant role in developmental differentiation. Moreover, *initial* polyploidy and aneuploidy do not change the main course of development. The striking morphological changes occurring during nuclear development in the Ciliates (Nanney, 1953; Sonneborn, 1954) probably have no direct parallel in the development of higher forms. Recently, characteristic enzymatic patterns have been established for nuclei in different tissues (See Allfrey *et al.*, 1955), and characteristic chromosomal changes associated with differentiation have been reported (Pavan and Breuer, 1952), but such studies fail to distinguish between nuclear differentiation as a consequence and nuclear differentiation as a causal factor in developmental alterations. Relatively few experimental procedures in fact permit examination of the heritability of nuclear modifications as opposed to the heritability of the cellular conditions as a whole. In spite of this, most attempts to develop models of differentiation have focussed attention on the cytoplasm.

Two kinds of evidence have chiefly been responsible for the belief that cellular differentiation is primarily a cytoplasmic function. The first of these was that leading to the establishment of the chromosome theory of

inheritance. The chromosomes were shown to be the vehicles of inheritance and the precise and equal distribution of the chromosomes at most cell divisions was demonstrated. Hence, the genetic (hereditary) characteristics of the nuclei must as a rule remain constant and hereditary differences characterizing cell lineages during development must have a cytoplasmic basis. It should be pointed out, however, that the observations contradict merely the concept of a *gross* partition of the chromosomal elements among differentiated tissues; they do not eliminate subtle metabolic or structural variations in the nuclei, or patterns of activation and inactivation of genetic loci without material loss. Cytoplasmic systems of heredity were postulated as the alternative to gross modification or segregation of chromosomal material, and without sufficient regard for other types of nuclear alteration.

The second kind of evidence was that obtained from studies in experimental embryology, particularly those of the kind conducted by Spemann (1938). Constriction of a fertilized amphibian egg into two portions—one with and one without a nucleus—allowed the multiplication of the nucleus for several cell divisions in one half of the egg. The subsequent escape of one of the daughter nuclei to the other half provided an excellent test for the hypothesis of heritable nuclear differentiation. If the nuclei had irreversibly differentiated during the early cleavage stages, development should be aberrant in the half of the egg with only one of the many daughter nuclei. This was not observed. Hence, it was concluded that irreversible (heritable) nuclear differentiation did not occur, at least during the early cleavage stages. Technical limitations prevented similar studies at later stages. Recently, however, Briggs and King (1953) have developed techniques for extending such studies. Nuclei (with some associated cytoplasm) have been removed from the cells of blastulae, gastrulae and later stages and injected into enucleated eggs. Their results with nuclei which have undergone relatively few divisions confirm Spemann's conclusions, but with successively later stages, fewer and fewer nuclei were found capable of sustaining normal development when transferred. Finally they have established the heritability of nuclear alterations through serial transfers of differentiated nuclei (Briggs and King, personal communication). If taken at face value these results require a rejection of the persistent totipotentiality of nuclei and a reassessment of the role of the nucleus in development. The model systems based primarily on cytoplasmic modifications may have less significance in developmental differentiation than usually supposed.

The first problem raised is the nature of the nuclear modification. Gross changes in nuclear constitution may be eliminated as not occurring with sufficient regularity or frequency. Secondly, traditionally conceived "mutations" must be rejected. If nuclear alterations are to account for any major part of cellular differentiation, they must differ from mutations—not in their heritability in general or in any one specific characteristic, but in a constellation of characteristics. 1) They must occur in much higher fre-

quency. 2) They must not be distributed randomly in time. 3) They must occur as specific responses to specific stimuli. 4) Their variety must be sharply circumscribed by the original genetic constitution of the cell. These characteristics do not imply that the nuclear alterations are extra-chromosomal or that they can in every case be easily distinguished from gene mutations, but only that—as a class—developmental modifications differ significantly from mutations in the usual sense of the word.

Several model systems of nuclear differentiation may be developed on the basis of the work in the Ciliates. These formulations rely heavily on efforts to develop coherent cytoplasmic systems and indeed show various degrees of integration with those systems. The first model to be examined is one which might be pertinent to the Group A caryonidal system in *P. aurelia*, or to that in variety 1 of *T. pyriformis* (figure 2A). The various genes controlling alternative potentialities would initiate a series of reactions resulting in gene products. (The actual number of genes involved is not relevant.) These gene products might then interact, within the nucleus, in a manner similar to that proposed by Delbrück (1949) for a cytoplasmic "steady state." The result of this antagonistic interaction would be the establishment of one series of reactions and the inhibition of the others. Some terminal or sub-terminal reaction product from the prevailing reaction series could be transmitted through the cytoplasm and translated into a phenotypic specificity at the surface of the cell. Since the antagonistic reactions are conceived as restricted to the nucleus, they should take independent courses in different nuclei developing in the same cell.

One modification transforms this system from that characteristic of the Group A varieties to that for the Group B varieties. (figure 2B.) Instead of restricting the antagonistic reactions to the nucleus, these could "overflow" into the cytoplasm and influence newly developing nuclei to imitate the same series of reactions. It should be pointed out that the efficiency of this system of inter-nuclear "communication" determines whether such systems can be identified as "caryonidal." If the transfer through the cytoplasm is perfectly efficient, the role of the nucleus is obscured and "inheritance" appears to be strictly cytoplasmic. The Group B system was in fact long considered such a system and only recently has the role of the nucleus been clarified. The serotype system in *P. aurelia* (Beale, 1954) shares many of the properties of the Group B mating type system. Although no evidence is thus far available to indicate caryonidal inheritance, this system may be conceptualized as a slightly more efficient model of the Group B pattern. One other feature of the serotype system also distinguishes it from the mating type system, that is, the relative ease of reversing a differentiation. This is not, however, a qualitative difference, since mating type instability *can* also exist for long periods of time.

In any case, no major problem arises in developing models of varying degrees of stability and the degree of stability of a system might well be influenced by the requirements of the cell. Thus a unicellular organism is

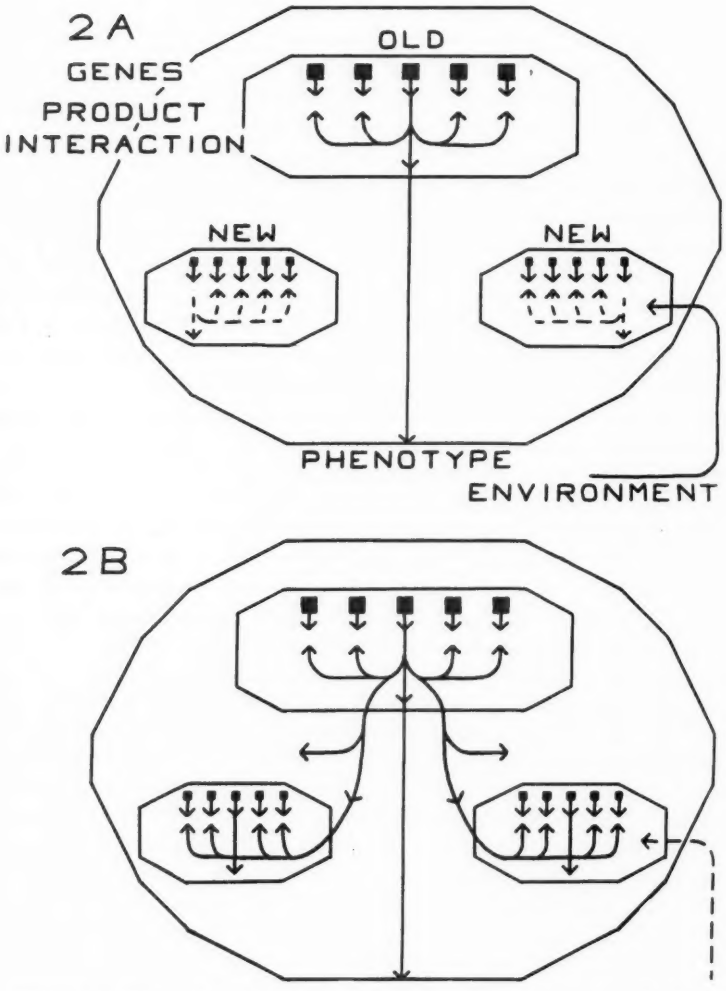


FIGURE 2. Two models of nuclear differentiation based on studies in the Ciliated Protozoa. (See Text)

to a large extent self sufficient, performing all the functions required for its existence; it cannot afford to relinquish as many potentialities as a metazoan cell, which participates in a system of symbiotic cellular interactions. Cellular differentiation in a unicellular organism in most cases would afford adaptation to particular vicissitudes in the environment, but the retention of plasticity—an ability to return to a former condition when the environment changed—would be beneficial. For this reason one might expect differentiation in unicellular organisms to be more freely reversible

than that in higher organisms. In the mating type system no hint is yet available concerning the selective advantage of a particular mating type for a cell considered as an independent entity, but all the advantages derive from the interactions of the cells in the population as a whole. In this particular case, therefore, selective advantage is contingent upon cellular interactions at the population level and not upon adaptability at the cellular level. Hence, the relative irreversibility of mating type differentiation may set it apart from most systems of differentiation in unicellular organisms and consequently may make it closer to systems of differentiation in metazoans.

These models do not involve alterations in the genes, the chromosomes or in their primary activities, but rather they assume steady state systems either localized within the nucleus or spilling over from the nucleus into the cytoplasm. It is of course conceivable that such a system could be entirely localized in the cytoplasm, in which case no evidence for nuclear differentiation would be available. On the other hand, alterations could occur at the level of the chromosomes themselves. Lederberg and Lederberg (1955) report evidence for persistant antigenic variations in Salmonella associated with reversible modifications at transducible genetic loci, and McClintock (1951) has studied what may be similar modifications in maize. It should be apparent, however, that *integrated* development, involving many different genetic loci, is scarcely conceivable without some form of intra-nuclear communication and coordination; steady state systems offer attractive possibilities in this regard. Fortunately, at this beginning of a reassessment of the role of the nucleus no immediate decision among the various models is required and no reason exists for believing that all nuclear differentiation involves the same mechanism.

Finally a word of caution is required regarding the organisms upon which these models are based. It is possible that the peculiarities of Ciliate structure provide patterns of cellular heredity far from typical. Most concern is developed in regard to the macronucleus, which differs in many respects from conventional nuclei. 1) It contains many sets of chromosomes, but their organization within the nucleus is an unsolved problem. The macronucleus may be composed of balanced sub-nuclei (Sonneborn, 1947a) or it may be a highly disorganized polyploid nucleus (Kimball, 1953). It must have mechanisms for the orderly distribution of genetic material at cell division but these are not understood. In any case, the problem of intra-nuclear coordination is more acute here than in simple nuclei and may have resulted in novel solutions. 2) The nuclear membrane in these forms does not break down at cell division as it does in most forms. This failure of breakdown may be conducive to the establishment of "nucleoplasmic" steady states which would be dissipated ordinarily at cell division. One might expect conventional cells to develop systems of cellular heredity more closely associated with the chromosomes—so as to persist through cell division—or to extend such systems more consistently through the

cytoplasm. These problems cannot be solved, however, until more is known about cellular heredity, not only in the Ciliates but in other organisms.

SUMMARY

The following features of caryonidal inheritance in variety 1 of *Tetrahymena pyriformis* are documented. 1. Cells with the same genetic constitutions may develop different phenotypes, which are inherited during vegetative growth. 2. Mating type diversities which arise at conjugation are distributed approximately at random among the products of the first fission after conjugation. 3. Within a genotype no positive correlation can be detected between the mating type of the parent and the mating type of the progeny.

Following Sonneborn, these and previously published observations, both on Tetrahymena and Paramecium, are interpreted as indicating nuclear differentiation as the basis for mating type determination in these forms. Suggestions regarding the mechanisms of nuclear differentiation and the general significance of such mechanisms are set forth.

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# THE SENSITIVITY OF THE GOLDFISH (*CARASSIUS AURATUS L.*) TO POINT HEAT STIMULATION

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## INTRODUCTION

It is known that fish can perceive temperature differences of .03 to .05 °C (Bull, 1937) and that they have thermoreceptors in the skin (Dijkgraaf, 1940). The state of our knowledge of temperature perception in fish has recently been summarized by Sullivan (1954). This review showed again that there are a number of questions which remain to be answered about the fish's perception of this important environmental variable. For instance: what is the level of precision of the individual receptor? Are fish more heat-sensitive on certain parts of their bodies than on others?

No specialization of nerve endings was found in the skin of those teleosts of which histological studies were made (Krause, 1928). There is, however, profuse branching of fine fibers with a great number of free endings per unit area.

In order to elucidate the mode of action of thermosensory surface receptors fish were tested for the distribution and relative density of these nervous elements.

These tests suggest that goldfish, typical teleosts, rely for their fine temperature (warmth) discrimination on areal summation which operates in several instances where the functioning of vertebrate warm and cold receptors has been studied in detail (Sand, 1938; Bullock, 1953; Hardy and Oppel, 1937; and Dodt and Zotterman, 1952). Dijkgraaf (loc. cit.) interpretated some of his observations on *Misgurnus fossilis* and *Ameirus nebulosus* in a similar manner.

## MATERIALS AND METHODS

The experiments involved a conditioned reflex training technique with which it could be ascertained whether or not the fish reacted to a point heat stimulus of a certain measurable intensity.

1. *Apparatus.* In a 30 gallon aquarium of 36 × 14 × 14 inches, an inner and outer chamber was built of opaque plastic. The outside of the tank was made opaque with window shade material.

A copper screen was placed on the bottom of the tank to form one electrode of an electric shocking system. Wooden frames with copper wire stretched over them functioned as the other electrode. They were fitted in the aquarium in such a manner that the experimenter could administer a mild

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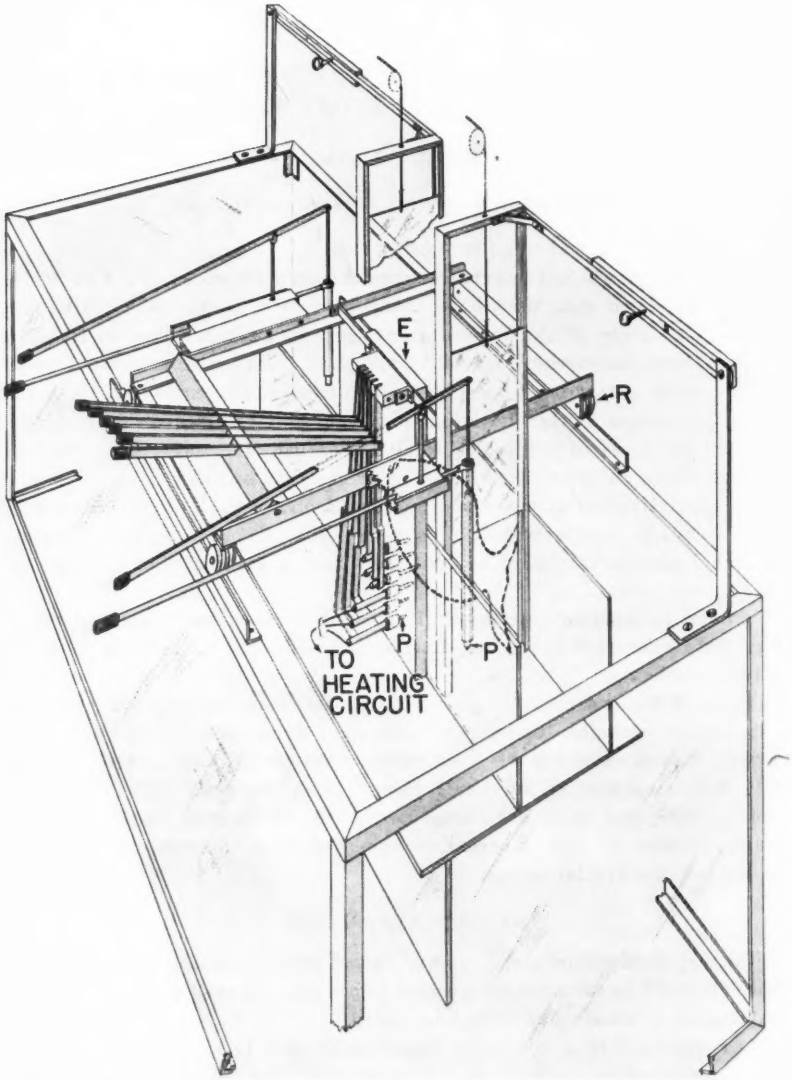


FIGURE 1. Experimental tank for spot heat stimulation of goldfish.
E. Experimental or test chamber.
P. Probes of nichrome wire.
R. Rail and rollers to shift probes along side of fish.

shock to the fish in any portion of the tank except the experimental chamber proper, which was situated in the middle of the long side of the tank distal to the observer (E, figure 1). There the heat stimulating devices made the placement of a shocking electrode difficult. The current used was 110

volts A.C., stepped down with a Variac transformer to about one to two volts depending upon the size of the fish. If a shock was to be administered in the experimental chamber itself, the current was increased slightly and the key activating the frame behind the fish was depressed.

A system of movable nichrome wire heating elements was arranged in the experimental chamber in such a way that a fish could be touched with the points of these heaters anywhere on its body (P, figure 1).

The heating elements were connected to a voltmeter and an ammeter on a selector switch circuit so that the heater wattage could be adjusted to any desired temperature with a potentiometer. Heat output curves at several wattages, from .5° to 40°C, were determined in well stirred water for each of the points of the heating probes by making contact with thermocouples of No. 30 B. and S. gauge copper constantan wire and a Leeds and Northrup Speedomax recording potentiometer. The entire arrangement of probes, and the levers to activate them were set on rails so that they could be moved along the side of the fish (R, figure 1). The action of the heating probes, each of about 2 sq. mm. surface, was individually controlled through a system of cams and levers from the outside of the aquarium.

A mirror covering the entire aquarium was placed above it at a 45 degree angle and a second smaller mirror was suspended so that the experimenter had a detailed view of the test chamber and could spot the position at which any one heating element touched the fish. The first step of the training procedure required bright light. A 100 watt light was hung over the aquarium and shaded to illuminate the tank only and leave the observer in the dark.

The heat stimulation of the various areas was carried out under subdued illumination. A 4 watt flourescent bulb was placed outside the experimental chamber slightly above the fish; it was kept on throughout the tests. The light emitted was sufficient to allow the experimenter to see in the mirror where the fish was touched but the fish presumably could not see the experimenter in the darkened room. Location of touch, temperature of probe and particulars about the behavior of the fish during the experiments were recorded on data sheets with fish outlines traced on them.

A foot operated buzzer from a doorbell was secured to the outside of the aquarium. It was to be used for a part of the training procedure.

2. *Training procedure.* A series of first order conditioned reflexes were used during training with the exception of one step that involved second order conditioning (table 1).

Initially the fish was trained to associate bright light with electric shock from which it found relief only in the test box. It could enter from the right or left so that eventually both sides of the fish were tested. As soon as the fish had entered the box, the overhead lights were turned out. At first, sliding doors confined the fish but soon it oriented itself on the door posts, even with the door open, and did not leave the box. As the next step in the training, the fish was touched very gently by advancing the unheated ni-

TABLE 1

TRAINING PROCEDURE FOR MAPPING OF HEAT RECEPTORS

| Stimulus | Animal Response |
|---|---|
| Light + Shock | into test box |
| Light | into text box |
| Touch | until no reaction |
| Heat (38–45°C) | out of test box |
| Touch | no reaction |
| Buzzer and Shock | out of test box |
| Buzzer | out of text box |
| Touch | still no reaction |
| Heat (1–4°C) + Buzzer | out of box or withdrawal of fin |
| Heat (1–4°C) | out of box or withdrawal of fin |
| (No response below ± 2°C) | |

chrome wire points toward it until it became used to the process and occasionally even leaned against the probes.

Egress from the test box was initially brought about by touch with a probe heated to register 38 to 45°C when submersed. In each trial, heat was applied only after one, two, three or four, and sometimes even five individual touches in random sequence so that the fish would not expect heat to follow touch after one probing. The rapid exit of the fish from the box at these heated touches lead one to believe that this level of temperature stimulation is noxious.

The fish were given a reward of three to five minutes rest outside the test box before the light was turned on again. In the next step of the training procedure, they were taught to associate the sound of the buzzer with an electric shock which served to drive them out of the test box. The habituation to unheated touch was, however, maintained during this part of the training until, (a) the fish always moved into the box on the bright light, (b) did not react to touches, and (c) would leave the box on the sound of the buzzer.

Only after the fish had learned these three different tasks and performed them well was unheated touch at times alternated with touch of a slightly heated probe. The temperature of this low heat stimulation varied from one to four degrees C. above that of the surrounding water which in turn fluctuated between 18 and 20 degrees during the course of the experiment. Since the fish were continuously kept in the same room they were acclimated to that temperature range and the water temperature was thus constant for any set of trials.

When the buzzer was sounded immediately following the gentle heat stimulation, it was expected that the fish would be able to extend this association to touch of low heat by a second order conditioned reflex from its recent training. Thus it was expected to associate buzzer with shock. If the animal could learn this, its response or lack thereof might give a clue of the threshold of heat stimulation for a small area on the surface of its body. Perhaps very few or even only one individual receptor unit could thus be made to respond. The results to be presently discussed below support this assumption.

The overall heat sensitivity of goldfish was ascertained in a separate set of experiments by a modification of Dijkgraaf's and Bull's (loc. cit.) techniques.

An important feature of these experiments, which will be reported in a separate paper, was the inclusion of one randomly chosen control among six fish at each trial.

Many observations of the control fish established that there was no extraneous clue and that the fish under test treatment always reacted to warm upwelling currents they had been taught to associate with a food reward.

The smallest heat increment reacted to was less than .1°C over a fraction of a second. The animals used for these tests were somewhat smaller than those used for spot stimulation, uniform in size, measuring about 11 cm. total length.

3. *The experimental animals.* Goldfish were chosen because of the ease in keeping and handling them. In the study on point heat stimulation, six animals were trained; they varied in size from eight to 18 cm total length and as far as could be ascertained from the scales were from two to eight years old. Complete training of a fish required up to a hundred trials and only five to seven of those could be performed per day. When more were attempted the animals became refractory. After training, 50 to 80 test trials were made, each again to be spaced not to exceed five to seven a day. Thus only three animals were put through the entire testing procedure, and it is on their performance that the following discussion is in the main based. The behavior of these few animals was so consistent that additional trials could hardly be expected to strengthen the evidence.

## RESULTS AND DISCUSSION

1. The fish were capable of forming the reflex associations required of them and their behavior on heat stimulation of all intensities was sufficiently distinctive to conclude that they were reacting to heat and not to touch. The difficulties encountered in the training make it appear likely, however, that several integrated first order conditioning processes and one second order conditioning represent a fish's limits of learning, at least in an artificial situation such as the one I employed.

2. Once the training had progressed sufficiently to work with the probes that were heated to four degrees or less over the surrounding water, the fish sometimes reacted to the warm touch on the fins with a withdrawal of the fin followed by a slight tremor. This, again, was never elicited by touch alone.

3. The smallest heat increment reacted to when applied gently but instantaneously on one area of 2 sq. mm was ±2°C. Heat sensitivity appeared to exist on the entire fish including the base, the surface, and some spots on the fringes of all fins.

No differences in sensitivity on any particular part of the body could be detected during the course of these experiments. It is possible that such

differentiation exists and that it could be demonstrated with a different or more refined method.

4. In 17 out of 150 stimulations the fish clearly gave no response to low heat between 2° and 4°C applied on their bodies; in an additional 13 trials the response was doubtful with eight of these doubtful responses occurring on the fringes of the fins while the rest was observed on the back and the posterior part of the head. In these cases the fish could be made to leave the experimental chamber only by substantial heat increments applied to the same area. Such "blank spots" on the body were less numerous than the areas on which low heat was perceived. They may have contained fewer temperature receptors than their surroundings or, and this is more likely, the failure of the fish to react was due to behavioral irregularities, to be expected in experiments of this kind. In the stimulation of the head region, I experienced considerable difficulty, probably because of the wider spread lateral line system which may have reacted to even the gentlest approach of probes and because the probes were moving close to the eye.

5. Although the heat sensitivity of the entire animal has already been mentioned under the training procedure, it should be emphasized that the fish reacted to a rapid change of ±0.1°C carried by convection currents. Slow and gradual changes of .2 degrees spread over two hours were ignored by the trained animals when they were selected as controls.

A low threshold in perception when temperature changes could be felt over the entire body surface, presumably exciting many or all receptors compared to a very much higher threshold of response to point heat stimulation is the result of areal summation in the performance of individual warm sensitive nerve endings in the skin. According to Krause's (loc. cit.) histological investigations there are in an area of about ⅛ of a square millimeter of a pike's skin, 7–10 free nerve endings derived from 2 or 3 fibers. None of these are detectably specialized and some out of this profusion (considering the entire surface of the fish) are probably warm or cold receptors while others react to touch. My experiments indicate that there are a few thousand unit warm receptors distributed over the body surface of the goldfish.

Before using the thermode of ± 2 $mm^2$, a pinpoint heater was tried. It had to be heated to register 10–15°C above the surrounding water, often reaching a noxious temperature level (see above) before the animal responded and frequently only after being touched for several seconds. The 2 $mm^2$ thermode, however, at 2°C and above brought reproducible and immediate responses. This suggests that the area subserved by a unit receptor—whatever the number of separate endings involved—is around 2 $mm^2$. It may be somewhat larger still if occurrence of "blank spots" (noted in 4 above) is taken into consideration.

In that case, one of our fish whose surface area on one side was 122 $cm^2$ might have around 6000 heat-sensitive spots on that side (blank areas were disregarded in this estimate).

The chance that warmth was registered even when the area adjacent to one or between several heat receptors was stimulated might tend to make this estimate too high; it is, however, safe to assume that a few thousand unit receptors are involved. It is probably a coincidence that this number is comparable to the total number of individual heat receptors estimated to exist in the rattlesnake pit organ (Bullock and Faulstick, 1953). Even though the number of nerve units involved may be comparable it should be mentioned that they are diffuse in the fish and concentrated in the pit of the rattlesnake. This arrangement as well as the possible amplification by accessory structures of the pit organ presumably facilitate central reactions to changes in the discharge rates of the nerves and explain the high heat discriminatory capacity of the snake.

In the human skin also the principle of areal summation has been demonstrated by Hardy and Oppel (loc. cit.). Here several hundred warm spots, when reacting simultaneously in an area of 200 $cm^2$ on the more heat sensitive areas of the body surface were capable of distinguishing much finer changes in heat energy flow than the goldfish and some other fishes which have been investigated (Bull, loc. cit.).

In connection with the 2°C found here as threshold stimulus on a small area, it may be interesting to note that Bohnenkamp and Schroer (1932) reported radiation of about 1.6°C to be the threshold for one warm sensitive spot on the forearm, while Bazzett and McGlone report .3°C at a rate of .2°C per second to suffice as stimulation on a single warm spot on the human prepuce.

The temperature sensitivity of Elasmobranchs which relies on the change in spontaneous discharge rates of the ampullae of Lorenzini has not yet been shown directly to rely on areal summation though the circumstantial evidence for this could hardly be more convincing (Sand, loc. cit.). Recently Hensel (1955) has extended Sand's observations by using a cathode ray oscilloscope.

Bullock (1954), stresses the fact that in every known case of detection of fine and rapid temperature changes areal summation of many—up to several thousand—receptor or receptor-small-fiber units seems to be involved and that, with few exceptions, 2°C seems to be close to the threshold for single receptors.

The secondary anatomy of the facial pit of a rattlesnake (Bullock, loc. cit.) endowed the animal not only with extreme sensitivity but also enables its owner to sense direction of the impinging change in heat flow. It is radiation rather than convection that the snake reacts to but if one considers the relative importance of convection in water and the fact that a fish's prey is almost always in thermal equilibrium with the surroundings it becomes unlikely that a set of specialized directional heat analyzers should have evolved in fishes.

Lastly, it should be stressed that the temperature receptor systems cited for comparison have been shown to involve spontaneous nervous discharge,

with discharge frequencies changing drastically upon fast heat changes in the environment.

I have not yet investigated an isolated skin nerve preparation but on the evidence accumulated so far it seems reasonable to expect to find another instance of a continuously discharging system. The individual receptor or the receptor-small-fiber-unit only produces "noticeable" or sufficiently significant changes at relatively large changes in environmental temperature. As more units become involved, smaller changes at slower rates are reacted to.

The cautions advocated by Hensel (1952) on account of the central thresholds for temperature sensations apply to my experiments. For this reason it should be attempted to isolate a skin-nerve preparation in order to gain knowledge on the performance of the receptors themselves. Yet information on what constitutes the adequate stimulus for the functional combination of peripheral and central elements can only come from the whole animal.

## SUMMARY

By conditioned reflex training it was found that goldfish sense ambient temperature increments of about .1°C over their entire body surface. Even the fins bear warm sensitive nerve endings. The receptors apparently operate on the principle of areal summation with an instantaneous heat increment of 2°C as the lower limit to which a fish would react when an area of 2 sq. mm was stimulated with heated probes after it had been trained not to react to touch stimuli.

An estimate of the total number of units involved in relative temperature discrimination suggested that several thousand units are involved; this number is of a comparable order of magnitude to that found in other vertebrates with a similarly fine or finer level of heat discriminatory capacity. This order of magnitude of unit receptors is found, be they collected in special organs or scattered over the entire body surface.

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# THE INFLUENCE OF BREEDING HABIT ON THE OUTCOME OF NATURAL HYBRIDIZATION IN PLANTS[1]

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Introgression and polyploidy are the two most common results of natural interspecific hybridization in plants. A great deal of knowledge has been gained about each one of these phenomena. The controlling factors which determine whether a given natural hybrid will backcross to the parental species or give rise to allopolyploid derivatives have not, however, been clearly defined. The purpose of the following paragraphs is to record some observations on the relation between the life cycle and breeding behavior of the parental species and the course of developments subsequent to the formation of a natural hybrid.

With the exception of a single race of one species, every polyploid in Gilia subgenus Gilia (Polemoniaceae) is small-flowered and self-pollinating (Grant, 1953; Grant, Beeks and Latimer, 1956). A correlation between self-pollinating habit and polyploid constitution is found also in the tribe Madiinae (Compositae) and the genus Microseris (Compositae-Cichorieae), (Clausen, Keck and Hiesey, 1945; Chambers, 1955). If the small size of the flowers is a criterion of autogamy, the same correlation is exhibited in Mentzelia (Loasaceae), Escholtzia (Papaveraceae), and Clarkia (Onagraceae) (Thompson and Lewis, 1955; Lewis and Snow, 1951; Lewis and Lewis, 1955).

A feature common to the aforementioned phylads, or at least to the sections of them including the polyploids, is the possession of an annual life cycle. The tendency for polyploidy to become established more frequently in selfers than in outcrossers in annual groups may be related to the mode of origin of the polyploid condition.

The most common process by which the chromosome number is doubled in annual plants seems to be non-reduction in natural hybrids (Clausen, Keck and Hiesey, 1945). The chances of a union of two unreduced gametes produced by a diploid hybrid will be most favorable if the plant reproduces by self-pollination. A hybrid which is committed by the characteristics inherited from the parental species to a system of outcrossing might not be able to give rise to any polyploid derivatives in the space of a single short blooming season. Another hybrid capable of self-pollination might produce one or several polyploid zygotes in the same period of time.

Where the time available for reproduction by the hybrid is critical, as in an annual herb, the breeding behavior of the parental species will therefore operate as a selective mechanism controlling the origin of polyploids.

[1]This paper has resulted from a program of research aided by a grant from the National Science Foundation.

This mechanism will favor the formation of polyploid types under conditions of self-pollination, but not under conditions of outcrossing, and will thus lead to the correlation observed in several unrelated groups of annuals between high chromosome numbers and small size of flowers.

Natural hybrids are no doubt formed between species representing every type of breeding system. The outcome of the hybridization may differ, however, according to the breeding behavior of the parental species. The large-flowered outcrossing species of Gilia subg. Gilia are, with but a single exception (*Gilia ophthalmoides flavocincta*), diploid; and a majority of these species are affected by introgression. The small-flowered self-pollinating diploid species, on the other hand, are relatively uncontaminated by introgression, but have been the starting points of the various polyploid species. The hypothesis may be considered, therefore, that the result of natural hybridization in annual plant groups will tend to be introgression when this hybridization involves cross-fertilizing species, and polyploidy when it involves self-fertilizing species.

It is well known that the conditions for establishment of a polyploid condition are generally more favorable in perennial herbs than in annuals (Müntzing, 1935; Stebbins, 1938, 1950, ch. 9; Gustafsson, 1947, 1948). The time available for a union of unreduced gametes is longer in the perennial types and the process of somatic doubling is more apt to take place. The hybrid *Primula verticillata* × *floribunda*, to cite a classical example, did not give rise to the tetraploid, *Primula kewensis*, in the first year of its existence, and it is fair to assume that the polyploid might never have originated if the parental species had been annuals. These considerations account for the more frequent occurrence of polyploidy in perennial herbs than in annuals.

Biennials, monocarpic perennials, and other short-lived perennial herbs without effective means of vegetative propagation frequently exhibit less polyploidy than the most closely related groups of annuals. This is the case in comparisons of the biennial Gilias and Ipomopsises with the annual Gilias, the biennial with the annual sections of Mentzelia, and the perennial with the annual sections of Microseris (Grant, 1956; Thompson and Lewis, 1955; Chambers, 1955). Polyploidy is either absent or very rare in the short-lived perennial groups, but is fairly common in the related annual forms.

In Ipomopsis (Polemoniaceae) the cross-fertilizing biennial groups are apparently entirely diploid, whereas tetraploidy is known in the outcrossing perennial *I. multiflora*. Similarly in Gilia, polyploidy is found in the autogamous annuals, as already noted, and also in the cross-fertilizing perennial group centering on *Gilia rigidula*, but is unknown in the cross-fertilizing biennial *Gilia stenothyrsa* group (Grant, unpubl.).

The explanation of the relatively low frequency of polyploidy in the biennial and short-lived perennial herbs mentioned above may lie in their habit of maintaining a high level of outcrossing. In the perennial species of Ipomopsis and Microseris this outcrossing is promoted by self-incompat-

ibility, and in the biennial Mentzelias by the floral mechanism. The annual relatives of these groups are predominantly self-compatible and undergo considerable self-fertilization under natural conditions. A life cycle which is long enough to tolerate the luxury of obligate cross-fertilization, but which is too short to afford much scope for somatic doubling or the chance union of unreduced gametes produced by different individuals, evidently presents very unfavorable conditions for the appearance of polyploidy.

Narural hybridization between self-incompatible and highly outcrossing species of biennial and short-lived perennial herbs will thus be expected to lead most frequently to introgression and only rarely to the formation of new polyploid forms. This expectation is fully realized in the biennial sections of Ipomopsis, the species of which are extensively affected by introgression on the diploid level. Short-lived perennials with an autogamous breeding system, on the other hand, may be expected to give rise to polyploid derivatives of hybrids at least as frequently as autogamous annuals.

Still another outcome of natural hybridization, apomixis, is confined, so far as is known, to long-lived herbaceous and woody groups which are regularly cross-fertilized in consequence of self-incompatibility or dioecism (Gustafsson, 1948; Stebbins, 1950, ch. 10). Polyploidy and introgression also occur frequently in perennial herbs and woody plants with a cross-fertilizing habit of breeding. A variety of courses of development is thus open to a long-lived, vegetatively reproducing hybrid with an allogamous breeding system.

## SUMMARY

Where the time available for reproduction by a natural hybrid is critical, as it is in annual herbs, biennials, and short-lived perennials, the natural hybridization will tend to run toward polyploidy under conditions of self-fertilization and toward introgression under conditions of outcrossing. We accordingly find in some annual and biennial groups, where hybridization has occurred on an extensive scale, that polyploidy is closely associated with autogamy and introgression with outcrossing. In long-lived perennial plants these tendencies may be obscured and reversed by other factors. The association of allopolyploidy with an outcrossing breeding system, which was noted by Gustafsson (1947), is found chiefly in plants with a perennial habit and/or vegetative means of propagation.

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# FURTHER STUDIES ON THE REVERSAL IN THE SEEDLING HEIGHT DOSE CURVE AT VERY HIGH LEVELS OF IONIZING RADIATIONS

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In an earlier publication (Schwartz, 1954), a preliminary report was presented describing the effect of high doses of ionizing radiations on the germination and growth of maize seeds. The dose curves, plotting seedling height against dose, indicated that, whereas at the low dose levels there is the expected inverse relation, a point is reached after which the original trend is reversed and a further increase in radiation dose results in an increase in seedling height. The low point in growth occurs at approximately 125,000 r of gamma rays. A typical dose curve is shown in Fig. 1. The purpose of this paper is to present an hypothesis to explain this phenomenon and the results of experiments designed to test its validity.

In the earlier studies it was noted that, at the high dose levels, around 500,000 r, where the seedlings were turgid and looked perfectly normal, growth had ceased after about five days. Root-tip smears showed no division figures, which suggested that the growth of these seedlings was due entirely to the elongation of those cells already present in the embryo at the time of irradiation.

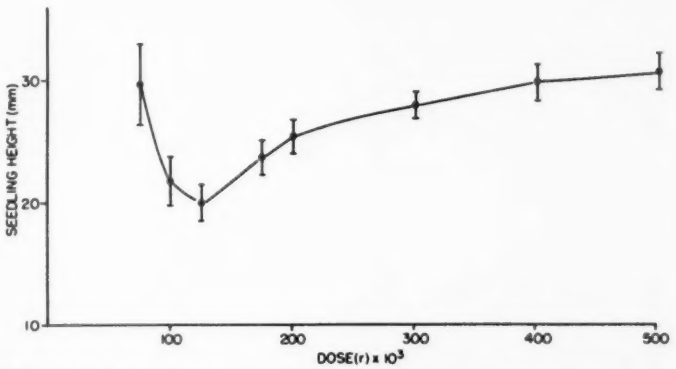


FIGURE 1. Relation between seedling height and gamma dose from $Co^{60}$ source. Confidence limits are 95 per cent. (From Schwartz, 1954.)

It is well established that there is a direct relation between dose of ionizing radiation and chromosome breakage; it is also known that ionizing

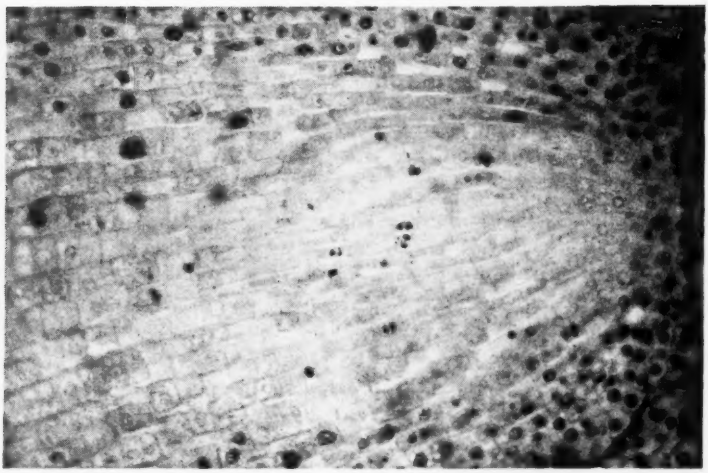

FIGURE 2. Longitudinal section through a young root tip from seed irradiated at 120,000 r of gamma rays.

radiations have an inhibitory effect on cell division (see Lea, 1947). Thus, with increased dose, as the average number of chromosome breaks per cell increases, the probability of mitosis is diminished. The genetic complement of a cell in which a large number of chromosome breaks have been induced will remain intact as long as the cell does not divide. Distribution

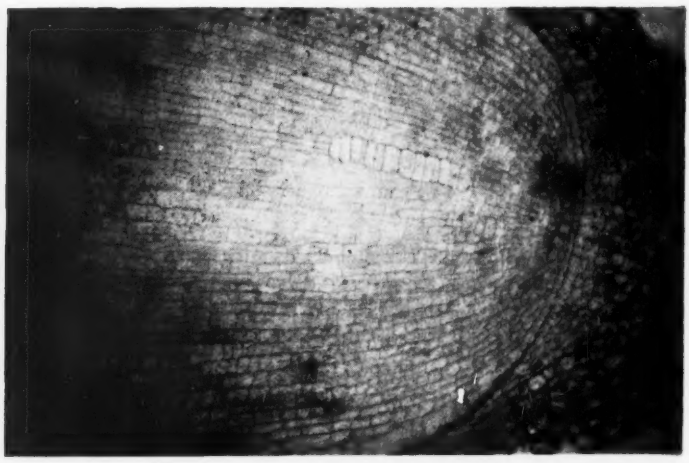

FIGURE 3. Section through young root from 500,000-r material showing complete absence of mitotic figures.

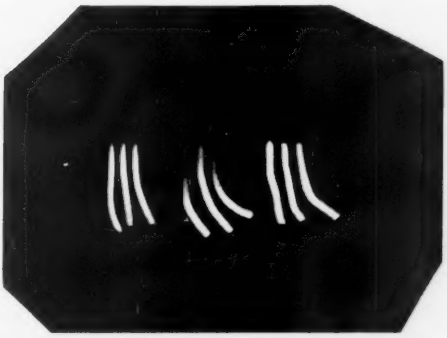

FIGURE 4. Root tips (tip up) from older seedlings with the following treatments: Left, control; middle, 120,000 r; and right, 500,000 r. The necrosis which is evident at 120,000 r does not appear in the 500,000-r material.

of its genes on many fragments rather than on whole chromosomes would not be expected to impair their function. However, because of aneuploidy, division will very likely result in death of the daughter cells. It is therefore proposed that, at the very high doses, the seedlings grow to their maximum height by elongation alone, with division being completely inhibited. As the dose is reduced to around 125,000 r of gamma rays, there is a de-

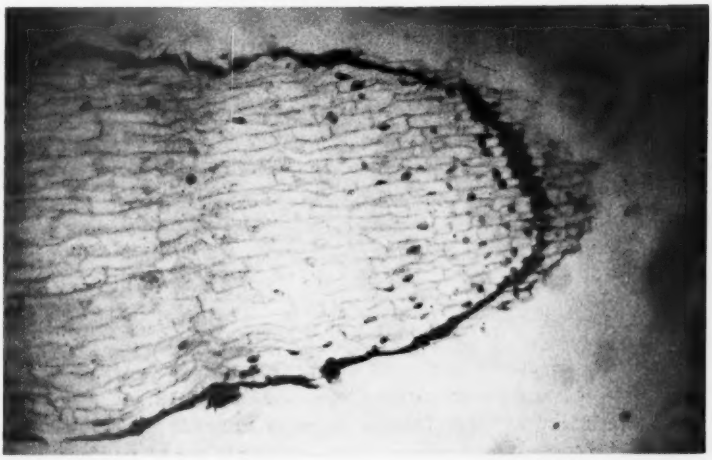

FIGURE 5. Section through older seedling root tip from the 500,000-r material. The cells at the tip have elongated and lost their meristamatic appearance.

crease in chromosome breakage, but an increase in cell lethality, since some of the damaged cells are now able to divide. As the dose is further reduced below 125,000 r, chromosome breakage is reduced to such an extent that even though the cells divide, lethality does not result and there is a marked increase in seedling growth. Cell division in the maize seedling is almost entirely confined to the region just below the coleoptilar node. It is to be expected that cell lethality in this region through which water and nutrients must travel to the plumule would have a drastic and very noticeable effect on the elongation and viability of cells in the higher regions.

On the basis of this hypothesis, certain predictions can be used to test its validity. The seedlings in the 125,000-r dose range, where the least growth is made, should show many more division figures than the taller, healthy-looking material at the higher doses. Also, a large amount of necrosis should occur in tissue in the region of cell division for the 125,000-r range seedlings and not at the higher doses. Both these predictions are borne out. There are still a fair number of cells in mitosis at 120,000 r, approximately one-third the number found in the control. Much chromosome damage, detected as bridges and fragments at anaphase, is evident at these doses (Fig. 2). No division figures were found in material that received doses in excess of 400,000 r (Fig. 3). The root tips used for these studies were removed from young seedlings whose roots had attained a length of approximately 1 cm and had not as yet shwon any signs of necrosis. Other seedlings were allowed to attain their maximum growth and were then examined for signs of cell lethality. Necrosis was very evident around the 125,000-r range, with none observed in the controls and at very high doses (Fig. 4).

The appearance of the root tips at the very high doses is worthy of some comment. Although they showed no sign of lethality, they were nevertheless quite abnormal in appearance, square rather than pointed and looked very much as though the root had been severed some distance above the tip. The cause of this abnormal appearance became apparent when these roots were sectioned and observed cytologically. Unable to divide, the cells in the tip region underwent elongation and maturation in spite of the large degree of chromosome breakage (Fig. 5) and lost their meristematic appearance.

## SUMMARY

The growth of seedlings irradiated as seeds with high doses is shown to be dependent on two main factors—chromosomal damage and rate of mitosis. The interaction of these factors is responsible for the reversal of the dose curve where seedling height is plotted against dose. At the very high doses, a point is reached where the effect of increased chromosomal damage is masked by the cessation of cell division.

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# LETTERS TO THE EDITORS

Correspondents alone are responsible for statements and opinions expressed. Letters are dated when received in the editorial office.

## A SECOND CASE OF TRANSLOCATION BETWEEN PATERNAL AND MATERNAL CHROMOSOMES*

Following X-ray treatment of adult *Drosophila melanogaster* males Sidky (1940) observed an unusual segregation of characters which he interpreted as the result of a translocation occurring during an early embryonic stage between the third chromosome derived from an irradiated sperm and the Y chromosome derived from an untreated egg of the attached-X mother. Although the evidence appeared to be decisive the interpretation has recently been questioned (Glass, 1955). Recently, during the course of an experiment in which translocations induced in mature sperm were detected, further evidence has been obtained to support Sidky's contention that rearrangement between paternal and maternal chromosomes can occur at an embryonic stage prior to polar cap formation.

Males of the "multipurpose stock" (Muller, 1954), $sc^8$.Y/y In49 *B*; $bw^D$, were mated to "multi-females", X.Y InEN *y*; *st*, and a 0.25% solution of a nitrogen mustard in 0.4% NaCl solution was administered to inseminated females as a vaginal douche. Translocations were detected by crossing $F_1$ males individually to virgin females like their mothers and the presence of a translocation among the Y, second and third chromosomes was indicated by the absence of recombinant classes involving the markers *y*, $bw^D$ and *st*.

In a single $F_2$ culture the following classes of offspring were obtained: $y^+bw^+st^+$ 19 ♂♂, *y* $bw^+$ $st^+$ 8 ♀♀, $y^+bw^Dst$ 16 ♂♂ and *y* $bw^Dst$ 11 ♀♀. The parental male was recovered and mated to three more multi-females. Again only the same four classes of offspring, with the exception of one non-disjunctionally-produced *y* $bw^D$ *st* ♂, were observed in a total of 165 flies. It appeared that a translocation between paternal and maternal second and third chromosomes had taken place. In order to determine whether the rearrangement involved the paternal second chromosome and the maternal third chromosome or vice versa the following crosses were made: (1) $F_2$ males phenotypically $y^+bw^Dst$, with the marker $bw^D$ which was derived from the original $P_1$ paternal second chromosome, and one of the st markers which was derived from the original $P_1$ maternal third chromosome, were mated to

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wild-type Oregon-R virgin females. If these males carried the translocated chromosomes, the latter would have been distributed to the $F_3$ offspring which were phenotypically $bw^D$ and upon subsequent crossing of these $F_3$ males to multi-females only the same four types of offspring as were observed in the $F_2$ would have appeared in the $F_4$ generation. (2) $F_2$ males phenotypically $y^+bw^+st^+$, with one of the unmarked second chromosomes which was derived from the original $P_1$ maternal second chromosome, and the $st^+$ third chromosome which was derived from the original $P_1$ paternal chromosome, were mated to virgin females with the genotype $y/y$; $^+/bw^D$ $st/st$. If now these males carried the translocated chromosomes, the latter would have been distributed to the $F_3$ offspring which were phenotypically $bw^Dst^+$ and upon subsequent crossing of these $F_3$ males to multi-females only the same four classes of offspring as were observed in the $F_2$ would have appeared in the $F_4$ generation.

When these two sets of crosses were made, $F_4$ cultures derived from $F_2$ $y^+bw^Dst$ males yielded the same four classes of offspring as noted in the $F_2$ while the $F_4$ cultures derived from $F_2$ $y^+bw^+st^+$ males yielded all recombinant types. Therefore the original translocation must have been either a reciprocal interchange between the paternal second chromosome and the maternal third chromosome or, less likely, since it would have required three breaks, a rearrangement of the deletion-insertion type between these chromosomes.

Most simply it could be assumed that a break induced in a sperm chromosome remained open until after fertilization at which time the broken pieces underwent interchange with induced or spontaneously broken pieces of an egg chromosome. With respect to mature sperm, in inseminated females at least, the evidence (Muller, 1940) is clear that breaks remain open until after fertilization. The evidence concerning breakage and rearrangements in oöcytes reveals a different situation. The main basis for his skepticism concerning Sidky's interpretation was the rarity with which translocations in females were found to occur, even following irradiation, in Glass's own work. But as Sidky was careful to point out, the occurrence of single chromosome breaks, spontaneous as well as induced, must be far more frequent than the recoverable eucentric interchanges which require the coincidence of two breaks and reunions. Furthermore, recent evidence (Herskowitz and Muller, 1953; Muller and Herskowitz, 1954; Herskowitz and Abrahamson, 1955) indicates that certain kinds of multi-break rearrangements, half-translocations, can be induced in females with a high frequency, and in addition breaks can rejoin prior to fertilization. With this latter point in mind it should be noted that in the present case the assumption is just as probable that even the initial breakage took place within the zygote, since the timing of chemically induced breaks is not limited to the period of external application of the agent. Whether the reason be the length of time necessary for mutagenically active particles to reach sites of breakage in the chromosomes or a "delayed-action", involving some sequence of chemical reactions that lead up to the linkages, it is possible for the entire se-

quence of breakage and reunion events to be delayed until a number of nuclear divisions after fertilization.

Consequently, unlike Sidky's original translocation between sperm and egg chromosomes, the present case cannot be cited as additional supporting evidence for the firmly established mechanism of interchange, viz., breakage first followed by reunion, or for delay until after fertilization of the union of pieces broken in mature sperm, but it does provide another example of a translocation between paternal and maternal chromosomes. However, both these cases do show that the paternal and maternal sets of chromosomes of Drosophila can become intermingled at a rather early stage, without that strict mutual isolation or "gonomery" which has sometimes been thought to occur.

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## PROBLEMS OF SEX IN UNICELLULAR ALGAE

In a recent paper published in German (1955a) and English (1955b), Hartmann has criticised an article of mine (Lewin, 1954), and I feel that a reply is called for.

Among the features which Hartmann seeks to criticise is the "partial" nature of my presentation. However, I did not set out "to give a correct critique of positive and negative findings concerning bipolar bisexuality," etc. My contribution to the 1951 symposium on "Sex in Micro-organisms" was limited to the subject of sex in unicellular algae, and I considered that it was outside my province to discuss other organisms. Nor did I aim to

write a comprehensive review, but rather to concentrate on "studies of cultures and experiments under controlled conditions, where a measure of reproducibility can be expected in the results" (Lewin, 1954).

If one of Hartmann's basic theses is that physiological differentiation is an *essential* condition for syngamy, then I feel I must continue to disagree. I know of no unequivocal evidence for intraclonal sex differentiation in any homothallic (haplomonoecious) unicellular alga, although Lerche's unpublished experiments with *Haematococcus* appear indicative. Even if such a condition were confirmed in one case, it might be rash to generalize.

I would prefer not to engage in polemics on priorities or on terminology. The terms "homothallism" and "heterothallism," as defined (with no claim for originality) in my article (p. 103), are in fairly general use in English-speaking countries (c.f. Whitehouse, 1949).

Finally, I might point out that an incomplete quotation from my article (p. 116: c.f. Hartmann, 1955a, p. 328; 1955b, p. 340) distorts the original meaning: and that, in his discussion of sex substances, Hartmann (1955a, pp. 326–328; 1955b, pp. 338–340) appears to have confused sex-substance activity of types 2 and 3. I make these corrections merely in the hope that further misunderstandings may not creep into the literature.

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It is good to have a reappraisal of our taxonomic group's early beginnings by this distinguished student of the protochordates. The author feels that the evidence suggests that it all started with neotenous ascidian larvae that invaded fresh water habitats. These gave rise to an unarmoured type of ostracoderm. Amphioxus is regarded as a degenerate descendant of the first chordates that is "not even a satisfactory vertebrate prototype." The hemichordates are 'not in any direct way relevant to the story.'

J. A. MOORE

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Diapause is defined as a state of arrested growth other than a mere quiescence brought on directly by an environmental factor. It may occur at any

of the major stages of the life history. The second chapter is devoted to a consideration of the influence of environmental factors, notably photoperiod, temperature, and food supply on the onset of diapause whereas the role of water is relegated to a subsequent chapter. Experiment shows that even in insects with obligatory diapause manipulation of the environment may affect the onset of the condition, and hence the occurrence of diapause is subject to selection. Of the environmental factors that terminate diapause temperature is the most important. The life cycles of parasitic insects are synchronized with those of their hosts, contain a period of diapause if this is present in the host, and also show adaptation to host races that differ as regards diapause. Available data on metabolism during diapause are discussed and there is also a chapter on hormonal control of diapause, with adoption of the schemes made familiar by the work of Williams and Wiggleworth. A final chapter discusses diapause as an adaptation to the environmental conditions to which an arthropod is exposed during its usual life cycle.

The book is essentially a review of the literature and as such is a valuable aid but in general lacks synthesis.

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